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PATENT & TRADEMARK OFFICE

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE	Application Number	09/914,046
	Filing Date	
	First Named Inventor	
	Group Art Unit	
	Examiner Name	
	Attorney Docket Number	2444-105
Title of the Invention: TARGETED LIPOSOME GENE DELIVERY		

DECLARATION

Assistant Commissioner for Patents
Washington, D.C. 20231

Dear Sir:

I, Esther Chang, declare that:

1. I am the same Esther Chang named as an inventor on the above-referenced patent application.

2. I received a B.A. degree in biology from Fu Jen University in Taiwan in 1968 and a Ph.D. in microbiology from Southern Illinois University in 1974. From 1982-1994 I held the positions of Assistant Professor, Associate Professor, and then Professor in the Department of Pathology, Uniformed Services University of the Health Sciences in Bethesda, MD. I also was a Research Professor in their Department of Surgery and the Director of their Tumor Biology Program. From 1994-1996 I held the position of Professor of Surgery (Research), Division of Otolaryngology/Head and Neck Surgery in the Department of Surgery

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at Stanford University Medical Center. Since 1996, I have held the position of Professor of Surgery (Consultant) there. I currently also hold the positions of Professor of Otolaryngology, Department of Otolaryngology/Head & Neck Surgery and Professor of Oncology, Department of Oncology, at the Georgetown University Medical Center, Lombardi Cancer Center, and have held those positions since 1996 and 1999, respectively. A copy of my curriculum vitae is attached hereto.

3. I have read the Office Action issued by the U.S. Patent and Trademark Office on November 28, 2004, and understand the grounds of rejection set forth therein.

4. In several rejections the examiner cited a paper by MacLean et al., and asserted that it discussed a method for coupling antibodies to liposomes in which an antibody or fragment thereof can be bound to the liposome via a sulfur atom that was part of a sulfhydryl group on the antibody or antibody fragment, thus indicating that she believed that the immunoliposome complexes claimed in the present application were taught in the prior art.

The discussion to which the examiner referred was part of a discussion of methods known in the art for coupling antibodies to liposomes, provided on page 329 of the MacLean et al. paper. Specifically, the method to which the examiner referred is method

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iii). MacLean et al. provided that succinimidyl-maleimide crosslinker could be used to attach antibodies containing sulfhydryl groups to liposomes, and that "Fab' antibody fragments already contain sulfhydryl groups but other antibodies may be modified using sulfhydryl generators," citing a 1983 paper by Duncan et al., a copy of which is attached hereto as Attachment 1.

5. The method taught by Duncan et al. would not be effective for binding an scFv fragment to a liposome. Duncan et al. teach introducing a sulfhydryl group onto an antibody by treatment with N-succinimidyl S-acetylthioacetate (SATA). SATA can alkylate any exposed amino group in the protein and does so randomly. This results in a so-called "Poisson distribution" of alkylated molecules. This means that only about 36% of the molecules are monosubstituted, about 33% have multiple substitutions and about 31% are unsubstituted. "Hence, single substitutions can be obtained with only a small fraction of IgG molecules and this is probably true for most proteins" (page 72, column 1). "Multiple substitutions would be expected in a significant proportion of the molecules" (page 72, sentence bridging columns 1 and 2). Moreover, there is no way of directing the substitution to a specific amino acid. This has two distinct negative implications for scFv fragments.

First, these multiple substitutions form polymers which will

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block the epitope on the scFv that binds to the receptor such that the desired immunoliposome complex cannot be formed. Second, even if such polymers don't form, there is no way to direct the binding of the antibody fragment to the maleimide-DOPE in the liposome. Thus, even if the scFv is only monosubstituted, it would only be by chance that the right amino acid would carry the substitution, such that the epitope that binds to the receptor would be exposed and available for binding to the liposome.

6. The authors of the Duncan et al. paper realize this drawback. They noted that "it is apparent that fewer potential polymerization sites will result if the single active group mentioned above is inserted into the peroxidase rather than the IgG" (page 72, column 3). Thus, they realize that this method does not work well for antibodies.

7. Both of these problems result in loss of biological activity. This does not happen in the method used in our application to produce scFv-liposome complexes. The DNA encoding the scFv is molecularly engineered such that a single -SH is introduced, the protein refolds properly to its natural state, and introduced -SH will not interfere with the binding of the antibody fragment to its receptor.

8. A further drawback to the approach taught by Duncan et al. is that before the antibody can be conjugated to The

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maleimide, The substituted protein has to be deprotected using either hydroxyl amine or an excess of another thiol which has to be "scrupulously removed before proceeding with the conjugation" (page 68, columns 1-2). These increased manipulations also will result in decreased biological activity.

9. Thus, this method is not useful for the conjugation of scFv to cationic liposomes. This is evidenced by the fact that although Duncan et al. published their paper in 1983, no scFv directly conjugated to cationic liposomes had been reported in the art as of the date we filed our patent application over 15 years later. The prevailing wisdom at the time of our invention was that scFv simply could not be used for immunoliposomes because they were simply too small and too fragile and efforts to modify them to provide a group which could react with a reactive group of the liposome would result in the loss of biological activity.

10. I further declare that all statements made herein of my own knowledge are true, and that all statements made on information and belief are believed to be true, and that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Codes, and that such willful false statements may jeopardize the validity of the application and any patent issuing

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thereon.

Esther Chang, Ph.D. 5/31/05
Date

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CURRICULUM VITAE

10/7/2002

PERSONAL

Name: Esther H. Chang
Place of Birth: Chungking, China
Citizenship: U.S. Citizen
Marital Status: Married with 1 daughter (Harford)
Work Address: Departments of Oncology & Otolaryngology
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EDUCATION

Fu Jen University, Taiwan	B.A.	1968	Biology
Southern Illinois University	Ph.D.	1974	Microbiology

PROFESSIONAL APPOINTMENTS

Trainee
U.S. Naval Medical Research Unit No. 2
Taiwan
1967 - 1968

Research Assistant
Southern Illinois University
1968 - 1971

Teaching Assistant in Immunology and Virology
Southern Illinois University
1971 - 1972

Research Associate
Southern Illinois University
1972 - 1973

Special Dissertation Fellow
Southern Illinois University
1973 - 1974

Visiting Fellow
National Institutes of Health
1974 - 1977

Visiting Associate
National Institutes of Health
1977 - 1978

Cancer Expert
National Cancer Institute
1978 - 1982

Assistant Professor
Department of Pathology
Uniformed Services University of the Health Sciences
1982 - 1983

Associate Professor and Coordinator for Medical Genetics Curriculum
1983 - 1988

Department of Pathology Uniformed Services University of the Health Sciences Professor, Department of Pathology Research Professor, Department of Surgery Coordinator for Medical Genetics Curriculum Director, Tumor Biology Program Uniformed Services University of the Health Sciences	1988 - 1994
Professor of Surgery (Research) Division of Otolaryngology/Head & Neck Surgery Department of Surgery Stanford University Medical Center	1994 - 1996
Professor of Surgery (Consultant) Division of Otolaryngology/Head & Neck Surgery Department of Surgery Stanford University Medical Center	1996-Present
Professor of Otolaryngology Department of Otolaryngology/Head & Neck Surgery Georgetown University Medical Center Lombardi Cancer Center	1996-Present
Professor of Oncology and Otolaryngology Departments of Oncology and Otolaryngology Georgetown University Medical Center Lombardi Cancer Center	1999-Present

HONORS AND OTHER SPECIAL RECOGNITION

Honor Society of Phi Kappa Phi	1972
Special Dissertation Fellow Southern Illinois University	1973 - 1974
Author, two papers in 100 most-cited papers in Life Sciences, Current Contents, November 5, 1984	1982 - 1983
Conference Organizer-International Conference on Molecular Biology of Neoplasia Taipai, Taiwan	1984
Ad Hoc Reviewer for NIH Study Section	1985
One of six awardees, Visiting Scholar Exchange Program, National Academy of Sciences, American Council of Learned Societies and Social Science Research Council	1986 - 1987
Member, Merit Review Committee, USUHS	1987 - 1989
Ad hoc Member, Review Panel for Assessment of Department of Energy research projects on chemical toxicology	1989
Member, Faculty Senate Education Committee, USUHS	1990 - 1991
Member, Editorial Board of Antisense Research and Development	1990 - Present

Member, Steering Committee on Prescribing of Drugs by Military Psychologists	1991
Chairman, Subcommittee for Faculty Resources for the Educational Program, Institutional Self-Study at USUHS	1991 - 1993
Member, Scientific Advisory Committee on Design Study for Life Span Experiments in Mice on Carcinogenesis and Biological Effects of Heavy Charged Particles, NASA	1992 - 1994
Chairman, Subcommittee to Examine Faculty, Middle States Association Reaccreditation Self-Study, USUHS	1992 - 1993
Ad hoc Member, Special Review Committee, Epidemiology, NCI	1992
Author, one Nature paper in top ten most cited papers in medicine Science Watch, September, 1992	1992
Member, Board of Scientific Counselors, Division of Cancer Biology, Diagnosis and Centers, National Cancer Institute	1993 - 1995
Member, NASA Life and Microgravity Sciences and Applications Advisory Committee	1994 - Present
Member, Interim ad hoc Board of Scientific Counselors, National Cancer Institute, NIH	1995 - 1996
Chair, Molecular Genetics Study Section, U.S. Army Breast Cancer Research Program	1997
Chair, Experimental Gene Therapy, Program Committee AACR Annual Meeting	1999
Member, Board of Scientific Advisors, National Cancer Institute	1999 - 2004
Member , Editorial Board of Cancer Gene Therapy	1999 - Present
Member, Scientific Program Committee. Chair, Gene Therapy Program NCI-EORTC-AACR Symposium	1999
Distinguished Alumni, Fu Jen University	1999
10th Lecturer, Stewart Lectureship	2000
Member, NASA Focus Group - National Academy of Sciences, Committee on Science, Engineering, and Public Policy	2000
Member, Committee of Scientific Advisors, United States Military Cancer Institute	2001 – Present
Ad hoc member, Experimental Therapeutics I + II, Study Section, NIH	2002
Organizer, Conference on "Tumor Specific Delivery by Non-Viral Systems" Maui, Feb. 2003 Sponsored by NCI	2002-2003
Approximately 10 annual invited lectures at national and international conferences and academic and research institutes	1982 - Present

DISSERTATION TITLE

Comparative Studies of Growth Patterns of Ganjam Virus in CE, BHK and VERO and *Aedes albopictus* Cells

RESEARCH ACTIVITIES

Undergraduate

Insect tissue culture. Studied growth pattern of insect line cells (Bombyx, Aedes and Antheraea) and adapted two lines into hemolymph-free media. Gained some experience in the growth of Japanese Encephalitis Virus in insect cells and newborn mice.

Graduate School

Arboviruses (Togaviruses). Electron microscopy. Compared the growth of VSV in insect cells and chicken embryo fibroblasts. Determined the viral RNA profiles in each cell line.

Characterized Ganjam Virus, an ungrouped arbovirus.

Postgraduate

RNA tumor viruses - interferon effect. Studied interferon's inhibitory effect on the replication of murine leukemia virus. (In Robert M. Friedman's laboratory, National Institutue of Arthritis, Metabolic and Digestive Diseases, NIH).

Molecular genetics. Cloned and characterized murine leukemia and sarcoma viruses. Investigated the origin and the functional organization of Harvey murine sarcoma virus. Molecularly cloned four DNA fragments containing human homologous sequences of v-*ras* (2 Harvey and 2 Kirsten) and demonstrated their oncogenic potentials. Studied potential human oncogenes. (In Douglas R. Lowy's Laboratory, Dermatology Branch, National Cancer Institute, NIH).

Current

1) Molecular genetic basis of familial cancer syndrome and the involvement of human oncogenes and tumor suppressor genes in carcinogenesis.
2) Modulation of oncogene expression by sequence-specific antisense oligonucleotides.
3) Molecular basis of cellular radioresistance and radioprotection.
4) Tumor Suppressor Gene Therapy for Cancer (Head and Neck, Breast and Prostate)
5) Ligand directed, tumor-targeted liposome-based systemic gene delivery

MEMBERSHIP IN ORGANIZATIONS AND PROFESSIONAL AFFILIATIONS

Honor Society of Phi Kappa Phi	1973-
American Association for the Advancement of Science	1983-
Society of Chinese Bioscientists in America	1988-
The Wound Healing Society	1991-
American Association for Cancer Research	1993-
American Society of Gene Therapy	1997-

PUBLICATIONS - ESTHER H. CHANG

1. R. M. Friedman, **E. H. CHANG**, J.M. Ramseur and M.W. Myers. Interferon-directed inhibition of chronic murine leukemia virus production in cell cultures: Lack of effect of intracellular viral markers. **J. Virol. 16**: 569-574 (1975).

2. R. M. Friedman, J.C. Costa, J.M. Ramseur, M.W. Myers, F.T. Jay and **E. H. CHANG**. Persistence of the viral genome in interferon-treated cells infected with oncogenic or nononcogenic viruses. **The J. Infectious Diseases 133**: A43-A50 (1976).

3. R. M. Friedman, F. T. Jay, **E. H. CHANG**, M. W. Myers, J. M. Ramseur, S. J. Mims, T. J. Triche, and P.K.Y. Wong. Interferon-directed inhibition of chronic murine leukemia virus production in cell cultures. In: Control of Neoplasia by Modulation of the Immune System. (M.A. Chirigos, ed.), Raven Press, New York (1977), pp. 347-359.

4. R. M. Friedman, E. F. Grollman, **E. H. CHANG**, L. D. Kohn, G. Lee and F. T. Jay. Interferon and glycoprotein hormones. In: Texas Reports on Biology and Medicine (1977), pp. 326-329.

5. R. M. Friedman and **E. H. CHANG**. Interferon action. Possible mechanisms of antiviral activity. In: Interferons and Their Actions (M. Stewart, ed.) CRC Handbook Series (1977), pp. 145-152.

6. **E. H. CHANG**, S. J. Mims, T. J. Triche, and R. M. Friedman. Interferon inhibits mouse leukemia virus release: An electron microscope study. **J. Gen. Viron. 34**: 363-367 (1977).

7. P. K. Y. Wong, P. H. Yuen, R. Macleod, **E. H. CHANG**, M. W. Myers, and R. M. Friedman. The effect of interferon on *de novo* infection of Moloney murine leukemia virus. **Cell 10**: 245-252 (1977).

8. **E. H. CHANG**, M. W. Myers, P. K. Y. Wong, and R. M. Friedman. The inhibitory effect of interferon on a temperature-sensitive mutant of Moloney murine leukemia virus. **Virology 77**: 625-636 (1977).

9. **E. H. CHANG**, and R. M. Friedman. A large glycoprotein of Moloney leukemia vrius derived from interferon-treated cells. **Biochem. Biophys. Res. Commun. 77**: 392-398 (1977).

10. **E. H. CHANG,** F. T. Jay and R. M. Friedman. Physical and morphological alteration in the membrane of AKR cells following interferon treatment and their correlation with the establishment of the antiviral state. **Proc. Natl. Acad. Sci. 75**: 1859-1863 (1978).

11. **E. H. CHANG,** E. F. Grollman, F.T. Jay, G. Lee, L. D. Kohn and R.M. Friedman. Membrane alterations following interferon treatment. In: Human interferon. W. Alton Jones Cell Science Center, Lake Placid, New York (1978), pp. 85-99.

12. A. K. Bandyopadhyay, **E. H. CHANG,** C. C. Levy and R. M. Friedman. Structura abnormalities in murine leukemia viruses produced by interferon-treated cells. **Biochem. Biophys. Res. Commun. 87**: 983-988 (1979).

13. G. L. Hager, **E. H. CHANG,** H. W. Chan, C. F. Garon, M. A. Israel, M. A. Martin, E. M. Scolnick and D. R. Lowy. Molecular cloning of the Harvey sarcoma virus closed circular DNA intermediates: Initial structural and biological characterization. **J. Virol. 31**: 795-809 (1979).

14. H. W. Chan, C. F. Garon, **E. H. CHANG**, D. R. Lowy, G. L. Hager, E. M. Scolnick, R. Repaske and M. A. Martin. Molecular cloning of the Harvey sarcoma virus circular DNA intermediates: II. Further structural analyses. **J. Virol. 33**: 845-855. (1980).

15. A. I. Oliff, G. L. Hager, **E. H. CHANG,** E. M. Scolnick, H. W. Chan and D. R. Lowy. Transfection of molecularly cloned Friend murine leukemia virus DNA yields a highly leukemogenic helper independent type C virus. **J. Virol. 33**: 475-486 (1980).

16. S. L. Berger, M. J. Hitchcock, K. C. Zoon, C. S. Birkenmeier, R. M. Friedman and **E. H. CHANG**. Characterization of interferon messenger RNA synthesis in namalva cells. **J. Biol. Chem. 255:** 2955-2961 (1980).

17. E. **H. CHANG**, J. Maryak, D. M. Wei, T. Y. Shih, R. Shober, H. L. Cheung, R. W. Ellis, G. L. Hager, E. M. Scolnick and D. R. Lowy. Functional organization of the Harvey murine sarcoma virus genome. **J. Virol. 35**: 76-92 (1980).

18. R. W. Ellis, D. DeFeo, J. M. Maryak, H. A. Young, T. Y. Shih, **E. H. CHANG,** D. R. Lowy and E. M. Scolnick. A dual evolutionary origin for the rat genetic sequences of Harvey murine sarcoma virus. **J. Virol. 36**: 408-420 (1980).

19. **E. H. CHANG** and D. R. Lowy. Transformation by molecularly cloned Harvey murine sarcoma virus DNA. **J. Supramol. Struc. 9 (Supp. 4)**: 237 (1980).

20. E. M. Scolnick, T. Y. Shih, J. Maryak, R. Ellis, **E. H. CHANG** and D. Lowy. Guanine neucleotide binding activity of *src* gene product of rat-derived murine sarcoma viruses. **Ann. N.Y. Acad. Sci. 354**: 398-409 (1980).

21. **E. H. CHANG**, R. W. Ellis, E. M. Scolnick and D. R. Lowy. Transformation by cloned Harvey murine sarcoma virus DNA: Efficiency increased by long terminal repeat DNA. **Science 210**: 1249-1251 (1980).

22. D. DeFeo, M. A. Gonda, H. A. Young, **E. H. CHANG**, D. R. Lowy, E. M. Scolnick and R. W. Ellis. Analysis of two divergent rat genomic clones homologous to the transforming gene of Harvey murine sarcoma virus. **Proc. Natl. Acad. Sci. 78**. 3328-3332 (1981).

23. D.R. Lowy, R.W. Ellis, D. DeFeo, **E. H. CHANG**, M.A. Gonda, H.A. Young, N. Tsuchida, T.Y. Shih and E.M. Scolnick. The cellular p21 sarc genes represent a family of divergent normal genes which have the capacity to transform mouse cells. In: RNA Tumor Viruses, New York, Cold Spring Harbor (1981), p. 294.

24. D.R. Lowy, R.W. Ellis, D. DeFeo, **E. H. CHANG**, M.A. Gonda, A. Young, T.Y. Shih and E.M. Scolnick. The family of cellular P21 sarc genes. In: Intl. Union of Microbiol. Soc., Virology Division (1981), p. 462.

25. **E. H. CHANG**, D.R. Lowy, M. Gonda, D. DeFeo, E.M. Scolnick and R.W. Ellis. The p21 gene family: Human and rodent DNA sequences homologous to the transforming genes of Harvey and Kirsten murine sarcoma viruses. In: Advances in Comparative Leukemia Research (1981), pp. 379-380.

26. D. R. Lowy. **E. H. CHANG**, R. W. Ellis, D. Defeo and E. M. Scolnick. Elevated levels of an evolutionarily conserved normal rat protein can induce cellular transformation. **Clin. Res. 29(2):** 428 (1981).

27. S. K. Chattapadhyay, **E. H. CHANG**, M. R. Lander, R. W. Ellis, E. M. Scolnick and D. R. Lowy. Selective amplification of *onc* genes in mammalian species. **Nature 296**: 361-363 (1982).

28. D. R. Lowy, **E. H. CHANG**, R. M. Ellis, M. A. Gonda, T. Shih, D. DeFeo and E. M. Scolnick. Harvey and Kirsten sarcoma viruses and the P-21 gene family. **J. Cell Biochem. Suppl. 6**: 194 (1982).

29. **E. H. CHANG**, M. A. Gonda, R. W. Ellis, E. M. Scolnick and D. R. Lowy. The human genome contains four genes homolgous to the transforming genes of Harvey and Kirsten murine sarcoma viruses. **Proc. Natl. Acad. Sci. 79**: 4848-4852 (182).

30. **E. H. CHANG**, M. A. Furth, E. M. Scolnick and D. R. Lowy. Tumorigenic transformation of mammalian cells induced by a normal human gene homologous to the oncogene of Harvey murine sarcoma virus. **Nature 297**: 497-483 (1982).

31. D. R. Lowy, M. A. Gonda, M. E. Furth, R. W. Ellis, E. M. Scolnick, and **E. H. CHANG.** Tumorigenic transformation of mammalian cells induced by elevated levels of a normal human *onc* protein. **Clin. Res. 30(2)**: 421 (1982).

32. C. J. Tabin, S. M. Bradley, C. L. Borgmann, R. A. Weinberg, A. G. Papageorge, E. M. Scolnick, R. Dhar, R. Lowy and **E. H. CHANG**. Mechanism of activation of a human oncogene. **Nature 300**: 143-149 (1982).

33. B. D. Crawford, **E. H. CHANG**, J. L. Goodwin, C. E. Hildebrand, P. M. Kraemer, J. L. Longmire and R. D. Palmiter. **J. Cell Biochem. Suppl. 7**: 135 (1983).

34. **E. H. CHANG**, M.A. Gonda, M.E. Furth, J.L. Goodwin, S.E. Yu, R.W. Ellis, E.M. Scolnick and D.R. Lowy. Characterization of four members of the p21 gene family isolated from normal human genomic DNA and demonstration of their oncogenic potential. In: Gene Transfer and Cancer, Raven Press, New York (1983), pp. 189-197.

35. **E. H. CHANG**, M.A. Gonda, E.M. Scolnick and D.R. Lowy. Characterization of 4 divergent human genomic clones homologenes to the transforming p21 genes of Harvey and KiMuSV. In: Gene to Protein--Translation into Biotechnology, American Press (1983), p. 512.

36. D.R. Lowy, M.A. Gonda, M.A. Furth, R.W. Ellis, E.M. Scolnick and **E. H. CHANG**. The human genes homologous to p21 *ras* viral oncogenes. In: Tumor Viruses and Differentiation, Alan R. Liss, Inc., (1983), pp. 435-444.

37. D. Samid, **E. H. CHANG** and R.M. Friedman. Revertants from interferon-treated mouse cells transformed by a human oncogene. In: The Biology of the Interferon System, Elsevier Science Publishers, (1983), pp. 359-360.

38. M. S. McCoy, J. J. Toole, J. M. Cunningham, **E. H. CHANG**, D. R. Lowy and R. A. Weinberg. Characterization of a human colon/lung carcinoma oncogene. **Nature 302**: 79-81 (1983).

39. S. J. O'Brien, W. G. Nash, J. L. Goodwin, D. R. Lowy and **E. H. CHANG**. Dispersion of the *ras* family of transforming genes to four different chromosomes in man. **Nature 302**: 839-842 (1983).

40. M. R. Pincus, J. van Reswoude, J. B. Harford, **E. H. CHANG** and R. D. Klausner. Prediction of the three-dimensional structure of the transforming region of the EJ/T24 human bladder oncogene product and its normal cellular homologue. **Proc. Natl. Acad. Sci. 80**: 5253-5257 (1983).

41. S. J. O'Brien, W. G. Nash, R. Bauer, **E. H. CHANG** and L. J. Seigel. Trends in chromosomal and oncogene evolution in vertebrates. "Uses and Standardization in Vertebrae Culture Cells" (M. K. Paterson, ed.), *IN VITRO* **Monograph No. 5**: Gaithersburg Tissue Culture Association (1984), pp. 204-214.

42. D. Samid, **E. H. CHANG** and R. M. Friedman. Biochemical correlates of reversion in interferon-treated mouse cells transformed by a human oncogene. **Biochem. Biophys. Res. Commun. 119:** 21-28 (1984).

43. D. Samid, **E. H. CHANG** and R. M. Friedman. Inhibition by interferon of transformation induced by a human *ras* oncogene. **Biochem. Biophys. Res. Commun. 126(1):** 509-516 (1985).

44. D. Samid, Z. Schaff, **E. H. CHANG** and R.M. Friedman. Reduction in *ras* expression accompanies phenotypic reversion of interferon-treated, c-Ha-*ras* oncogene transformed mouse cells. In: The Biology of the Interferon System (H. Kirchner and H. Shellekens, eds.), Elsevier, Amsterdam (1985), pp. 189-198.

45. D. Samid, Z. Schaff, **E. H. CHANG** and R. M. Friedman. Interferon-induced modulation of human *ras* oncogene expression. Endocoids. In: Progress in Clinical and Biological Research, Vol. 192 (H. Lal, F. La Bella and J. Lane, eds.), Alan R. Liss, New York (1985), pp. 265-268.

46. D. Samid, **E. H. CHANG** and R.M. Friedman. Specific inhibition by interferon of oncogene-induced transformation. In: Serono Symposia Publications, Vol. 24 (F. Dianzani and G.B. Rossi, eds.), Raven Press, New York, (1985), pp. 425-422.

47. D. Samid, D.M. Flessate, J.J. Greene, **E. H. CHANG** and R.M. Friedman. Mechanisms of Antioncogenic activity of interferon in the 2-5A System: Molecular and clinical aspects of the interferon-regulated pathway. In: Prigin. Clinical and Biological Research, Vol. 202, (B.R.G. Williams and R.H. Silverman, eds.), Alan R. Liss, New York (1985), pp. 203-210.

48. D. Samid, **E. H. CHANG** and R.M. Friedman. Regulation of *ras*-expression by interferon. In: Proc. Asian Congress Pharmacol., (1985), pp. 343-364.

49. **E. H. CHANG**, J.K. Lin, and P. C. Huang, eds. Molecular Biology of Neoplasia. Academia Sinica, 1985

50. **E. H. CHANG**. Viral and cellular oncogenes. In: Molecular Biology of Neoplasia. (E.H. Chang, J.K. Lin and P.C. Huang, eds.) Academia Sinica - Taipei, Taiwan (1985), pp. 191-203.

51. D. Samid, **E. H. CHANG**, and R. M. Friedman. Biological and morphological characteristics of phenotypic revertants appearing in interferon-treated mouse cells transformed by a human oncogene. **J. Exp. Path. 2(3)**: 211-222 (1985).

52. **E. H. CHANG**, P. L. Morgan, E. Lee-Lawlor, K. Pirollo, E. A. White, P. N. Tsichlis and D. H. Patrick. Pathogenicity of retroviruses containing either normal human c-Ha-*ras* 1 or bladder carcinoma EJ/T24 *ras* gene. **J. Exp. Path 2:** 177-190 (1985).

53. R. L. Stallings, R. Black, B. D. Crawford and **E. H. CHANG**. Assignment of *ras* protooncogenes in Chinese hamster: Implications for linkage conservation. **Cytogenet. Cell Genet. 43:** 2-5 (1986).

54. **E. H. CHANG**, R. Black, T. Masnyk and J.B. Harford. Effect of interferon on growth of A431 cells and expression of EGF receptors. In: Advances in Gene Technology: Molecular Biology of the Endocrine System. (D. Puett, *et al*, eds.), Proc. 18th Annual Miami Winter Symposium, 1986, pp. 370-371.

55. **E. H. CHANG**, R. Black, Z.Q. Zou, T. Masnyk, J. Ridge, P. Noguchi and J.B. Harford. Interferon modulates growth of A431 cells and expression of EGF receptors. In: Interferons as Cell Growth Inhibition and Antitumor Factors. (R.M. Friedman, T. Merigan and T. Sreevalsan, eds.), Alan R. Liss, New York (1986), pp. 335-350.

56. **E. H. CHANG**. Oncogenes and familial cancer syndrome. CAPA 86 Conference Proceedings, College Park, MD, 1986, pp. 21-29.

57. **E. H. CHANG**, K. F. Pirollo, Z. Q. Zou, H. Y. Cheung. E. L. Lawlor, R. Garner. E. White, W. B. Bernstein, J. F. Fraumeni, Jr. and W. A. Blattner. Oncogenes in radioresistant, non-cancerous fibroblasts from a cancer-prone family. **Science 237:** 1036-1039 (1987).

58. **E. H. CHANG**, J. Ridge, R. Black Z. Q. Zou, T. Masnyk, P. Noguchi and J. B. Harford. Interferon-induces altered oncogene expression and terminal differentiation in A431 cells. **Proc. Soc. Exp. Biol. Med. 186:** 319-326 (1987).

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117. Y. J. Jang, K.F. Pirollo, Z. Hao, Y. Chiang, and **E.H. CHANG**. Restoration of the G_1 Block and Apoptotic Pathway in SCCA of the Head and Neck by Adenoviral Vector Mediated p53 Gene Therapy. **Submitted to Carcinogenesis.**

118. L. Xu, K.F. Pirollo, W.H. Tang, L.M. Xiang, A. Rait, D. Ulick, W.A. Alexander and **E.H. CHANG**. Systemic P53 Gene Therapy Using a Tumor-Targeted Adenoviral Vector Results in Radio/Chemo Sensitization and Long-Term Tumor Regression. **Submitted to Science.**

119. A. Rait, K.F. Pirollo , L. Xu, V. Rait, L. Xiang and **E.H. CHANG**, Antisense HER-2 Oligonucleotides Sensitize Human Breast Cancer to Taxotere *In Vitro* and *In Vivo.* **Submitted to Human Gene Therapy.**

120. K B. Bouker, K.F. Pirollo and **E.H. CHANG**, p53: Culprit or Bystander in the Treatment Failure of Radio/Chemotherapy. **Submitted to JNCI.**

121. M.S. Jhaveri, A.S. Rait, J.B. Trepel, **E.H. CHANG**. Antisense oligonucleotides targeted to the human alpha folate receptor sensitize breast cancer cells to doxorubicin treatment *in vitro*. **Submitted to Molecular Cancer Therapeutics.**

THESIS AND DISSERTATION

1. E. H. CHANG. Adaptation of Grace's continuous lines of insect cells to medium containing heterologous serum. Bachelor's Thesis (U.S. Naval Medical Research Unit No. 2, Fu Jen University, Taipei, Taiwan (1968).

2. E. H. CHANG. Comparative studies of growth patterns of Ganjam Virus in CE, BHK and VERO and *Aedes albopictus*cells. Ph.D. Dissertation, Southern Illinois University, Carbondale, Illinois (1974).

PATENT - APPLICATION FILED

1. c-Raf Transgenic Non-Human Mammals.
2. An Automated Method for the Detection of p53 Mutations.
3. Treatment of Tumors by a Combination of Radiation Therapy and Transduction with Polynucleotide Encoding Wild Type p53.
4. Method of Reversal of Resistance to Radiation Therapy and to Chemotherapy in Cancer Cells Using Sequence-Specific Anti-HER-2 Oligonucleotides.
5. Modified Antisense Nucleotides Complementary to a Section of the Human Ha-*ras* Gene.
6. Targeted Liposome Gene Delivery.
7. Compositions and Methods for Reducing Radiation and Drug Resistance in Cells.
8. Systemic Viral/Ligand Gene Delivery System and Gene Therapy.
9. Ligand-PEG "Post-coated" Cationic Liposomes for Targeted Gene Delivery.
10. Antibody Fragment-Targeted Immunoliposomes for Systemic Gene Delivery.
11. A Simplified and Improved Method for Complexing an Antibody Fragment-Targeted Immunoliposome for Systemic Gene Delivery.

RESEARCH GRANTS

Esther H. Chang, Ph.D.

1. Currently Active Support:

1. National Institutes of Health, A Novel Improvement on Radiotherapy for SCCHN
   P.I. 20% Effort on Project
   Project Period: 1 APRIL 1999-31 MARCH 2003
   Total: $286,320

2. Natl. Foundation for Cancer Research, Chemosensitization of Breast Cancer by Systemic Delivery of Anti-HER2 Oligonucleotides
   P.I. 5% Effort on Project
   Project Period: 1 OCTOBER 2000- 30 SEPTEMBER 2003
   Total: $130,435

3. NIH STTR Phase I Application 1R41 CA91660-01A1. Targeting Stealth™ Liposome for Cancer Gene Therapy.
   Jointly with SynerGene Therapeutics, Inc
   10% Effort on Project
   Requested project period: 1 JUNE 2002 – 31 MAY 2003
   Total Requested: $41,143 (Georgetown portion).

4. NIH STTR Phase II Application 2R42 CA80449-2A1 Immunoliposome-Mediated Gene Therapy for Prostate Cancer.
   Jointly with SynerGene Therapeutics, Inc.
   20% effort on project.
   Requested Project Period: 1 SEPTEMBER 2002 – 31 AUGUST 2004
   Total Requested: $169,280 (Georgetown University Portion).

5. NCI, decision Network Program, Transferrin-Liposome (Synerlip) Mediated Systemic Gene Delivery for Human Prostate Cancer.
   P.I.
   Project Period: February 1999-
   The Decision Network has chosen our transferrin-lioposome-p53 complex (Synerlipp53) for further development and testing in Phase I clinical trials by the NCI.

6. NCI, Rapid Access to Intervention Development (RAID) Program, Tumor-Specific Targeting of wtp53 by Anti-Transferrin Receptor Single Chain Antibody: A New Therapeutic Strategy for Prostate Cancer Treatment
   P.I.
   Project Period: 1 APRIL 1999-
   The RAID program does not supply funds to the approved projects. The RAID is designed to accomplish tasks that are rate-limiting in bringing discoveries from the laboratory to the clinic. Thus, in support of this project the RAID program is producing, through the use of NCI's development contracts, GLP/GMP grade reagents including the TfRscFv, the liposome and the wtp53 expression plasmid.

2. Past Support:

1. USUHS, Regulation of the Expression of Human c-ras Genes.
   1 OCTOBER 1982 - 30 SEPTEMBER 1985.
   $60,000 - 3 years. P.I. 10%

2. USUHS, Molecular Cloning of a Tumor Oncogene in a Cancer-Prone Family.
   1 OCTOBER 1985 - 30 SEPTEMBER 1989.
   $154,125 - 4 years. P.I. 10%

3. NIH, Oncogenes (c-ras) in Human Cancer Induction.
   1 MAY 1983 - 30 APRIL 1986.
   $160,000 - 3 years. P.I. 40%

4. Medical Applications of Advanced Laser Technology (MAALT). Probing the Molecular Mechanisms of Carcinogenesis.

1 JANUARY 1986 - 31 DECEMBER 1988.
$150,000 - 3 years. P.I. 10%

5. NIH, Oncogenes in Human Cancer Induction.
1 SEPTEMBER 1986 - 31 DECEMBER 1989.
$258,791 - 3 years. P.I. 40%

6. NIH, a program project. Subproject III. Modulation of Tumor Cell Growth. Program project P.I. Paul O. P. T'so, Johns Hopkins University. Program project. Title: Oligonucleotide Analogs as Antiviral/Anticancer Agents.
1 AUGUST 1986 - 31 DECEMBER 1989.
$145,190 - 3 years. Co-P.I. 15%

7. Medical Applications of Advanced Laser Technology (MAALT). Experimental Therapy of Human Colorectal Tumors.
1 JANUARY 1989 - 31 DECEMBER 1990.
$80,000 - 3 years. P.I. 10%

8. NIH, Modulation of Tumor Growth in vitro and in vivo.
1 JULY 1990 - 30 JUNE 1995.
$649,018 - 5 years. P.I. 15%

9. NIH, Oncogenes in Human Cancer Induction.
1 DECEMBER 1989 - 30 NOVEMBER 1994.
$757,798 - 5 years. P.I. 25%

10. USUHS, Inherited Genetic Defects in Li-Fraumeni Syndrome.
1 OCTOBER 1992 - 30 SEPTEMBER 1995.
$81,000 - 3 years. Co-P.I. 5%

11. Naval Medical Research and Development Command. Demonstration of Cytokines and Growth Factors in Wound Healing.
1 APRIL 1991 - 30 SEPTEMBER 1996
$485,400 – 5.5 years P.I. 5%

12. National Foundation for Cancer Research, HU0001, Modulation of the Radiation-Resistant Phenotypes of Tumor Cells by Sequence-Specific Oligonucleotides.
1 OCTOBER 1988 - 30 SEPTEMBER 1999
$638,750-9 years P.I. 10%

13. NIH, CA45158, The Status of Suppressor Genes in a Cancer-Prone Family.
1 DECEMBER 1994 - 30 NOVEMBER 1999
$1,003,887 – 5 years P.I. 30%

14. Genetic Therapy Inc./NOVARTIS, Sensitization of Tumors to Radiation Therapy by Restoration of the G1 Checkpoint.
1 OCTOBER 1997 - 30 SEPTEMBER 1998
$60,000-1 year P.I. 5%

15. NIH STTR Phase I Application 1 RA1 CA80449-01. Immunoliposome-Mediated Gene Therapy for Prostate Cancer.
(Jointly with SynerGene Therapeutics, Inc.)
1 NOVEMBER 1998 - 31 OCTOBER, 1999
$57,600 (Georgetown University Portion) 1 year, P.I. 10%

16. NIH, 5D50 CA58185-06, SPORE in Breast Cancer (Marc E. Lippman, P.I.).
Development Project, p53 Mediated, Tumor-Targeted Sensitization to Chemotherapy and Radiotherapy.
1 SEPTEMBER 1997 - 31 AUGUST 2001
$50,000- 4 year P.I. 10%

17.DOD Concept Award, Systemic Apoptin Gene Therapy for Chemo/Radiosensitization of Breast Cancer
1 SEPTEMBER 2000-31 AUGUST 2001

$50,000- 1 year P.I. 5%

3. Pending Support

1. NIH RO1 Application. Non-Invasive Methods to Assess p53 Gene Therapy Effects.
   Submitted on February 1, 2001
   15% effort on project
   Request Project Period: 1 DECEMBER 2001 – 30 NOVEMBER 2005
   Total Requested: $1,513,200

2. NIH RO1 Application. Surrogate End-Points to Assess p53 Therapy in SCCHN.
   Submitted on June 1, 2001
   10% effort on project (CO-PI)
   Requested Project Period: 1 April, 2002 – 31 March, 2006
   Total Requested: $1,496,640

3. NIH RO1 Application. Systematic Sensitization of Pancreatic Cancer to Gemzar
   Submitted on February 1, 2002
   20% effort on project
   Requested Project Period: 1 December 2002 – 30 November 2006
   Total Requested: $1,864,810

ANALYTICAL BIOCHEMISTRY 132, 68–73 (1983)

A New Reagent Which May Be Used to Introduce Sulfhydryl Groups into Proteins, and Its Use in the Preparation of Conjugates for Immunoassay

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The Wellcome Research Laboratories, Langley Court, Beckenham, Kent. BR3 3BS, United Kingdom

Received December 1, 1982

A synthesis of the *N*-hydroxysuccinimide ester of *S*-acetylthioacetic acid is described. This material is stable when stored dry and has advantages over the currently available reagents used to introduce sulfhydryl groups into a variety of proteins. Proteins modified with this reagent can be used to prepare conjugates for enzyme immunoassay. The conjugation techniques described cause little or no loss of either enzyme activity or antibody titer and function, and the conjugates contain little polymeric material.

Sulfhydryl–maleimide coupling is a technique for the preparation of protein to protein conjugates which occurs through very stable amide and thioether bonds with the necessary modification reactions usually causing very little disruption to the functions of the proteins (reviewed by Ishikawa (1)). Proteins to be conjugated must have at least one functionally unessential sulfhydryl group; otherwise it is necessary to incorporate such a group into the molecule before conjugation is possible. There are three methods commonly used to achieve this: by reaction with *N*-succinimidyl 3-(2-pyridyldithio)propionate (SPDP; from Pharmacia), with methyl 4-mercaptobutyrimidate in the presence of 4,4'-dithiodipyridine (2), or with SAMSA[1] (3). Each of these methods introduces a sulfhydryl group protected from adventitious reaction, particularly oxidation, so that the substituted protein may be stored for long periods. Use of SAMSA has an advantage over the other methods in that deprotection is with hydroxylamine, rather than with excess of another thiol which has to be scrupulously removed before proceeding with the conjugation. SATA, the reagent described in this communication, has this advantage of SAMSA and two further. First, the electrostatic charge on the modified protein is less disturbed (loss of one positive charge per sulfhydryl inserted compared with the loss of a positive and gain of a negative). Second, only one form of derivative can be formed with SATA whereas the linking amide bond may be with either of the carboxyl functions of SAMSA, leading to problems in characterization of conjugates of small molecules and peptides.

MATERIALS

Succinimidyl 4-(*N*-maleimidomethyl)-cyclohexane-1-carboxylate was a gift from Dr. Ishikawa, or was obtained from Pierce–Warriner (Chester, U. K.). Horseradish peroxidase was Type VI from Sigma (London) Ltd., and had an R_z (4) of about 3. Thiopropyl Sepharose 6B and Sephacryl S-300 were from Pharmacia. Organic solvents were distilled and then stored over a Fisher Molecular Sieve, Type 4A, to ensure dryness. IgG was purified from sheep serum by salt fractionation and chromatography on DEAE-cellulose. Other chemicals were from BDH Poole (Dorset, U. K.) or Sigma (London) Ltd.

[1] Abbreviations used: SAMSA, *S*-acetylmercaptosuccinic anhydride; SATA, *N*-succinimidyl *S*-acetylthioacetate; IgG, Immunoglobulin G.

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:e Sulfhydryl Groups into
ugates for Immunoassay

GER WRIGGLESWORTH

ent, BR3 3BS, United Kingdom

icetic acid is described. This
rently available reagents used
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ugation techniques described
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:eding with the conjugation
eagent described in this com-
ias this advantage of SAMSA and
First, the electrostatic charge of
protein is less disturbed (loss of
charge per sulfhydryl inserted
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). Second, only one form of do
be formed with SATA whereas
nide bond may be with either of
functions of SAMSA, leading to
:haracterization of conjugates of
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MATERIALS

lyl 4-(*N*-maleimidomethyl)-cy-
:arboxylate was a gift from Dr.
was obtained from Pierce-War-
, U. K.). Horseradish peroxidase
from Sigma (London) Ltd., and
4) of about 3. Thiopropyl Se-
ınd Sephacryl S-300 were from
)rganic solvents were distilled and
over a Fisher Molecular Sieve,
ensure dryness. IgG was purified
serum by salt fractionation and
phy on DEAE-cellulose. Other
ere from BDH Poole (Dorset,
gma (London) Ltd.

METHODS AND RESULTS

(a) Synthesis of SATA. The structure of SATA is shown in Fig. 1. *S*-acetylthioacetic acid (2.68 g, 0.02 mol), made in 48% yield following the method of Benary (5), was dissolved in dry dichloromethane (5 ml), mixed with *N*-hydroxysuccinimide (2.30 g, 0.02 mol) in 30 ml of dichloromethane, and stirred at room temperature for 10 min. Dicyclohexylcarbodiimide (4.15 g, 0.02 mol) in 2 ml of dichloromethane was added and the reaction mixture was stirred for 18 h at room temperature. The precipitated dicyclohexylurea (4.024 g) was removed by filtration and the filtrate evaporated, then stirred with dichloromethane (50 ml). Residual urea (0.484 g; total recovery of the urea = 100%) was filtered off and the filtrate evaporated to give a yellow oil which crystallized on trituration with ether. The solid was recrystallized from 2-propanol to give SATA (3.162 g, 68%), mp 85–86°C; ν_{max} (KBr disk) 1825, 1787, 1740, 1695 cm^{-1}; δ ($CDCl_3$) 2.40 (S, 3H), CH_3CO; 2.77 (S, 4H) succinimidyl-; 3.93 (S, 2H) -S-CH_2CO-. (Found C, 41.41%; H, 3.84%, N, 5.70%; $C_8H_9NO_5S$ requires C, 41.56%; H, 3.90%; N, 6.06%).

The NMR spectrum of material stored for approximately 2.5 years at 4°C over silica gel showed about 10% decomposition to give *N*-hydroxysuccinimide. Further evidence of this slight decomposition is given by thin-layer chromatography using methyl ethyl ketone as solvent, and by small shifts in titration curves of the type shown in Fig. 2.

(b) Reaction of SATA with proteins. The protein to be alkylated with SATA was dissolved in 50 mM sodium–potassium phosphate, pH 7.5, containing 1 mM EDTA. Just before use, SATA was dissolved at concentrations up to 150 mM in dimethylformamide and added to the solution of protein at room temperature with the concentrations adjusted so that less than 10 μl of organic solvent were added/ml of aqueous solution. The reaction between 65 μM IgG and 520 μM SATA is complete within 10 min as may be shown by stopping the reaction (by the addition of Tris–HCl, pH 7.8, to 60 mM) at various times after mixing the reactants and measuring the extent of *S*-acetylthioacetylation of the protein. After derivatization the protein was separated from small molecules by gel filtration into the phosphate–EDTA, and was stored either at 4°C or frozen.

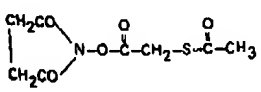

FIG. 1. The structure of SATA.

Particular proteins are *S*-acetylthioacetylated by SATA to an extent which depends on the concentrations of both reactants, and the relationship between these variables for certain proteins is shown in Fig. 2 and Table 1. The graph shown for IgG in Fig. 2 was obtained with IgG from sheep serum, but an identical relationship was found for a number of conventional and monoclonal IgG preparations. The effect of SATA treatment on the functional capability of an antibody was investigated using the mutant β-galactosidase-activating antibody system (6) which allows direct measurement of both the binding constant and stoichiometry of the antibody–enzyme reaction in solution. It was found that incorporation of up to a mean of at least 6 mol of *S*-acetylthioacetate/mol of IgG had no discernible effect on either of these constants, or on the extent of activation of the enzyme. Hence incorporation of this reagent into an antibody has little effect on its function. In six separate reactions of 65 μM IgG with 2.5 mol of SATA/mol of IgG an incorporation of a mean of 1.18 ± 0.06 (SEM) mol of sulfhydryl/mol of IgG was found after deacetylation (see below), and 24 ± 1.2% of the protein then failed to bind to activated thiopropyl Sepharose 6B during chromatography following the general method of Brocklehurst *et al.* (7). There was an unmeasurably low level of sulfhydryl in the unbound protein. (The concentration of unbound protein was measured after gel filtration to obviate interference from 2-mercaptopyridine from the column.)

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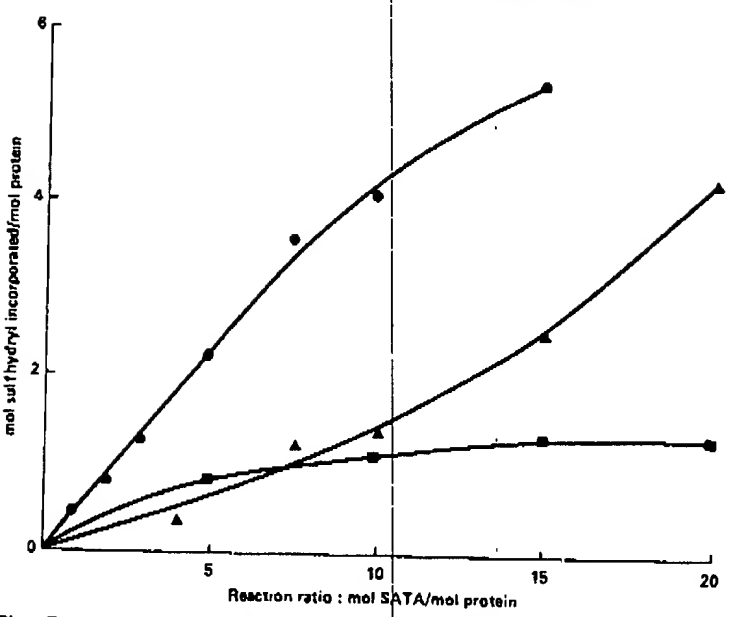

FIG. 2. The effect of the concentration of SATA on the modification of proteins. The mole ratios of SATA to protein shown on the abscissa were allowed to react with ■, 100 μM peroxidase; ●, 62.5 μM IgG; or ▲, 5.5 μM thyroglobulin. Sulfhydryl incorporation was measured as described in the text.

(c) Deacetylation of the derivative and measurement of sulfhydryl content. The acetyl group used to protect the sulfhydryl group during preparation of SATA and its incorporation into protein was removed with hydroxylamine (prepared by adding 50 mmol of hydroxylamine hydrochloride and 2.5 mmol of EDTA to about 80 ml of water, adding solid disodium hydrogen phosphate to give a pH of 7.5, then adjusting the volume to 100 ml, and using 100 μl of this solution for ea ml of protein solution to be deacetylated). The sulfhydryl was more than 90% expos after about 1 h at room temperature and there was negligible change in the sulfhydryl concentration over the next 24 h when the solution was kept stoppered at 4°C. Sulfhydryl was measured using Ellman's reagent (9) or 4,4'-dithiodipyridine (10) for solutions of he moproteins.

TABLE I

EFFECT OF IgG CONCENTRATION IN THE REACTION OF SATA WITH IgG

IgG concentration (μM)	Sulfhydryl/IgG (mol/mol)
60	4.0 ± 0.05
30	3.1 ± 0.2
12	1.84 ± 0.1

Note. A constant ratio of 10 mol of SATA/mol of IgG was allowed to react at various protein concentrations and the sulfhydryl inserted was then measured as described.

(d) Preparation of conjugates and their characterization. Reactive maleimide residues were incorporated into one of the proteins to be conjugated, usually IgG, by treatment with succinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (11), and the extent of reaction was measured by back-titration with 2-mercaptoethanol and Ellman's reagent. A titration curve similar to those in Fig. 2 enabled selection of the reagent concentration required for a given degree of substitution; for the conjugates described below the IgG was substituted with a mean of 4 maleimide residues/mol except as noted

The IgG derivative was
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Optical Density

FIG. 3. Chromatogra
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broken curve. Elution v

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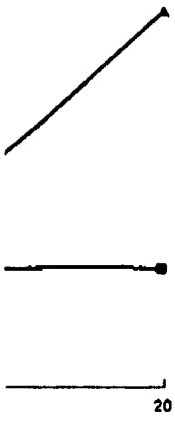

of proteins. The mole ratios of
0 μM peroxidase; ●, 62.5 μM
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Reactive maleimide resi-
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The IgG derivative was added over about 0.5 h to SATA-treated peroxidase (containing a mean of about 1 mol of sulfhydryl/mol of protein) that had been deacetylated previously. After 1–2 h at room temperature the reaction was stopped by addition of 2-mercaptoethanol to twice the original maleimide concentration and then, 15 min later, *N*-ethylmaleimide to twice the total sulfhydryl concentration. The ratio of peroxidase to IgG in the conjugates was calculated approximately from a knowledge of the absorbance of the conjugates at 403 and 280 nm and the R_z of peroxidase and it was found that the composition of the conjugate could be varied by changing the ratio of IgG and enzyme allowed to react or by varying the degree of maleimide substitution of the IgG. At a final IgG concentration of 20 μM, reaction of 12 nmol of the IgG with 38 or 200 nmol of monothiolated peroxidase resulted in conjugates containing a mean of 1.5 or 2.5 mol of enzyme/mol of IgG, respectively. Reaction of 20 nmol of IgG containing 9 mol maleimide/mol with 180 nmol of peroxidase yielded conjugates containing 5.4 mol of enzyme/mol of IgG.

The chromatographic and electrophoretic characteristics of a conjugate of IgG and peroxidase (350 nmol of peroxidase reacted with 110 nmol of IgG) are shown in Figs. 3 and 4. It can be seen that the conjugate was separated clearly from the excess of enzyme by chromatography on Sephacryl S-300. Electrophoresis of the pooled conjugate of Fig. 3 on polyacrylamide gels in the pH 2.4 system of Deibler *et al.* (12) (Fig. 4) shows that the conjugate is polydisperse but, unlike the conjugate prepared by periodate oxidation of peroxidase (13) and shown for comparison, has little high molecular weight material near the starting point of the gel, and no unlabeled IgG.

DISCUSSION

Our chief applications for SATA have been for the conjugation of an enzyme, usually peroxidase but on occasions urease, to IgG for use in enzyme immunoassay and for the conjugation of peptide hormones. For these applications it is important to minimize the formation of polymeric products so as to conserve the effective titer of the antibody and reduce nonspecific interactions. In order to

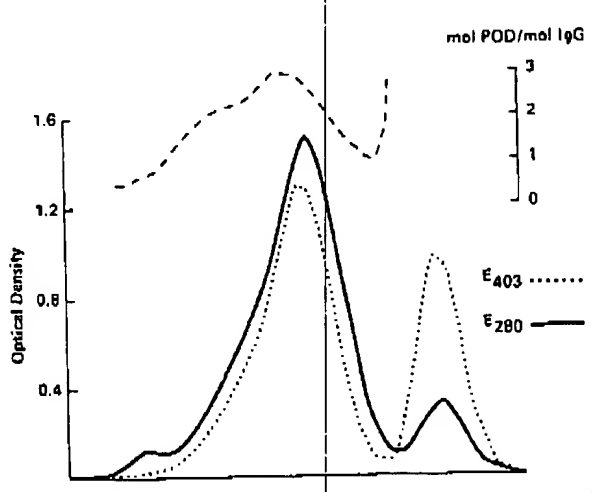

FIG. 3. Chromatography of an IgG-peroxidase conjugate. Conjugate (6 ml) was chromatographed on Sephacryl S-300 (95 × 0.8 cm²) in 50 mM Tris-HCl, pH 8.0, at a flow rate of 4 cm h⁻¹. The effluent was monitored at 280 and 403 nm, and the ratio of peroxidase to IgG in the eluate is shown by the upper broken curve. Elution volume increases toward the right.

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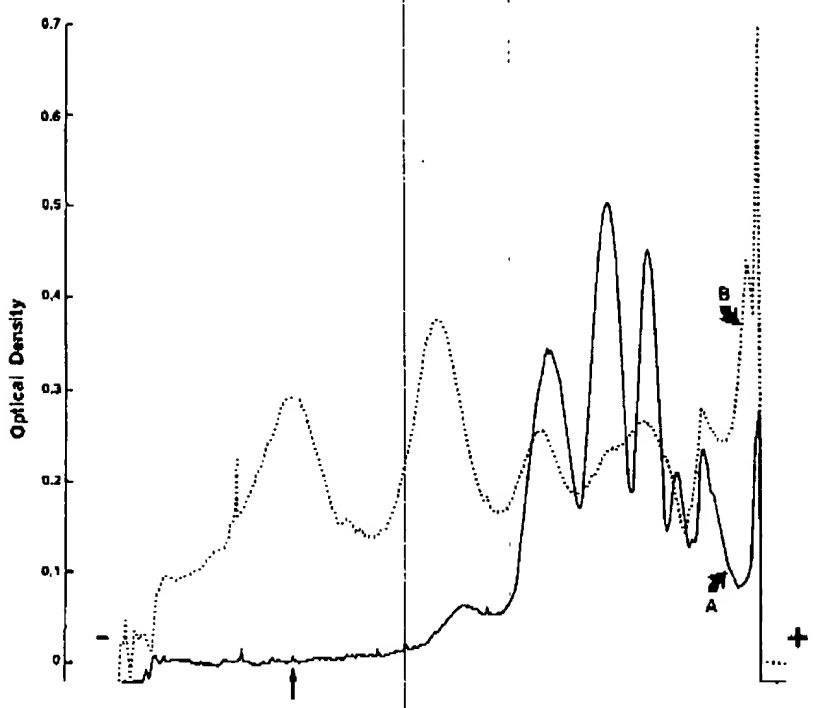

FIG. 4. Electrophoresis of IgG–peroxidase conjugates. About 30 μg of peroxidase conjugates ((A) from Fig. 3; (B) prepared by the Nakane and Kawaoi (13) technique) was electrophoresed for 10 mA·h/gel at pH 2.4 (12). The gels were stained with Coomassie blue R-250 then scanned at 595 nm. IgG runs to the point marked with an arrowhead. The top of the gel (anodic) is to the right.

ensure complete lack of polymer formation during conjugation, one of the proteins must bear only a single substituent. It would be expected that the reaction of SATA with IgG would give a distribution of alkylated molecules which is approximately Poisson in form: for IgG substituted with a mean of 1.18 mol of sulfhydryl/mol (as described in (b) above) a Poisson distribution would encompass some 36% of the molecules being monosubstituted, 33% having more than one substituent, and 31% being unsubstituted; this prediction is in reasonable agreement with the 24% found unsubstituted by experiment. Hence, single substitution can be obtained with only a small fraction of IgG molecules, and this is probably true for most proteins. Even with horseradish peroxidase in which very few amino groups are available for substitution ((14) and Fig. 2) multiple substitution would be expected in a significant proportion of the molecules. Nevertheless it is apparent that fewer potential polymerization sites will result if the single active group mentioned above is inserted into the peroxidase rather than the IgG. With this in mind we attempted to minimize the problem of polymer formation by slowly adding the IgG substituted with 4 mol of maleimide/mol. to a calculated molar excess of enzyme molecules bearing a mean of one substituent group. As can be seen from Fig. 4, this procedure is effective if the conjugation reaction is stopped after about 2 h. Conjugates prepared by this technique contain no unlabeled IgG, as may be seen from Fig. 4 and also by chromatography of conjugates of peroxidase on immobilized concanavalin A when no antibody is eluted before the enzyme. We have found that it is unnecessary to do more than dialyze the conjugation mixtures directly after

stopping the reaction
in order to prepare
a variety of enzyme
The synthesis of th
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Use of SATA is also
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ACKNOW

We thank Dr. Shireen
bovitis for discussion and
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REF

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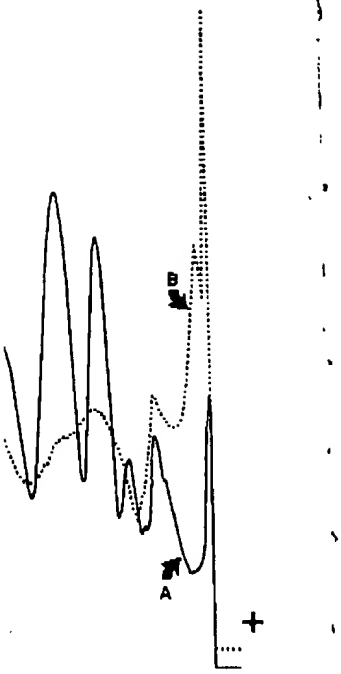

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stopping the reactions with *N*-ethylmaleimide in order to prepare products satisfactory for a variety of enzyme immunoassays.

The synthesis of this ester, SATA, is simple and gives a good yield of a stable product. Use of SATA is also straightforward and gives reproducible modification of a variety of proteins under mild conditions, with little loss of the activity or function of the protein.

ACKNOWLEDGMENTS

We thank Dr. Shireen M. Chantler and Mr. E. Krambovitis for discussion and for evaluation of our conjugates in their ELISA systems.

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Biochimica et Biophysica Acta 1237 (1995) 99–108

BBA Biochimica et Biophysica Acta

A new strategy for attachment of antibodies to sterically stabilized liposomes resulting in efficient targeting to cancer cells

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Received 21 October 1994; revised 8 March 1995; accepted 10 March 1995

Abstract

The development of long-circulating formulations of liposomes (S-liposomes), sterically stabilized with lipid derivatives of poly(ethylene glycol) (PEG), has increased the likelihood that these liposomes, coupled to targeting ligands such as antibodies, could be used as drug carriers to deliver therapeutic drugs to specific target cell populations in vivo. We have developed a new methodology for attaching monoclonal antibodies to the terminus of PEG on S-liposomes. A new end-group functionalized PEG-lipid derivative pyridylthiopropionoylamino-PEG-distearoylphosphatidylethanolamine (PDP-PEG-DSPE) was synthesized for this purpose. Incorporation of PDP-PEG-DSPE into S-liposomes followed by mild thiolysis of the PDP groups resulted in formation of reactive thiol groups at the periphery of the lipid vesicles. Efficient attachment of maleimide-derivatized antibodies took place under mild conditions even when the content of the functionalized PEG-lipid in S-liposomes was below 1% of total lipid. The resulting S-immunoliposomes showed efficient drug remote loading, slow drug release rates and increased survival times in circulation compared to liposomes lacking PEG. When antibodies recognizing several different tumor-associated antigens were coupled to the PEG terminus of S-liposomes, a significant increase in the in vitro binding of liposomes to the target cells was observed. The binding of S-immunoliposomes containing entrapped doxorubicin to their target cell population resulted in increased cytotoxicity compared to liposomes lacking the targeting antibody.

Keywords: Sterically stabilized liposome; Poly(ethylene glycol); Liposome targeting; Doxorubicin; Immunoliposome; Selective toxicity; Toxicity

1. Introduction

Site-specific delivery of drugs to diseased cells can lead to significant reductions in drug toxicity, and increased therapeutic effects. Therapeutic applications of liposomal drug delivery systems have been extensively explored in recent years [1–5], and several methods have been developed for attachment of antibodies (Ab) at the liposome surface [6–9] in attempts to target the liposomes in vivo to specific sites of drug actions. Attachment of Ab at the surface of classical formulations of liposomes results in their rapid removal from circulation by the cells of the mononuclear phagocyte system (MPS, also termed reticuloendothelial system) [10,11]. New formulations of sterically stabilized liposomes (SL) have been developed recently which have extended blood circulation times as a result of reduced rates and extents of uptake by MPS cells [12–15]. SL contain, as the key component, methoxy poly(ethylene glycol) distearoylphosphatidylethanolamine

Abbreviations: mPEG, methoxy poly(ethylene glycol); MPS, mononuclear phagocyte system; SL, sterically stabilized (Stealth®) liposomes; mPEG-DSPE, methoxy poly(ethylene glycol) distearoylphosphatidylethanolamine; PDP-PEG-DSPE, pyridyldithiopropionoylamino poly(ethylene glycol) distearoylphosphatidylethanolamine; SIL, sterically stabilized immunoliposomes; DSPE, distearoylphosphatidylethanolamine; SPDP, *N*-succinimidyl-3-(2-pyridyldithio)proprionate; DSC, disuccinimidylcarbonate; SC, succinimidylcarbonate; Boc, *tert*-butyloxycarbonyl; TLC, thin-layer chromatography; TEA, triethylamine; PDP, pyridyldithiopropionate; HSPC, hydrogenated soy phosphatidylcholine; SMPB, succinimidyl-4-(*p*-maleimidophenyl)butyrate; MPB-PE, maleimidophenylbutyrate-phosphatidylethanolamine; ^{3}H-CHE, [^{3}H]cholesteryl hexadecyl ether; ^{125}I-TI, [^{125}I]tyraminylinulin; Ab, polyclonal antibody; mAb, monoclonal antibody; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*'-(2-ethanesulfonic acid); Mes, 2-(*N*-morpholino)ethanesulfonic acid; CHOL, cholesterol; DTT, dithiothreitol; DXR, doxorubicin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; KLN 205, murine squamous lung carcinoma; Caov-3, human ovarian adenocarcinoma; HCT-15, human colon adenocarcinoma; mAb 174H.64, antibody recognizing KLN 205; mAb B43.13, antibody recognizing Caov-3; mAb M170, antibody recognizing HCT-15.

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(mPEG-DSPE) (4–8 mol% of the total lipid). This results in coverage of the lipid vesicle surfaces with grafted amphipathic polymer chains. It is believed that the high mobility of the mPEG chains, associated with their conformational flexibility, and also their water-binding ability, contribute to the steric stabilization which is responsible for their prolonged survival times in circulation [16,17].

We were interested in developing methods for attachment of Ab to the surface of SL which would meet a number of important criteria for 'ideal immunoliposomes'. These include: simplicity and ease of preparation, maintenance of prolonged circulation half-lives, retention of target recognition for the Ab, high coupling efficiency of the Ab to the liposomes, the ability to achieve high Ab densities at the liposome surface, the ability to achieve efficient drug remote loading, appropriate drug release characteristics, and compatibility with use in humans.

Attachment of Ab at the surface of SL, in the phospholipid headgroup region, can result in some decrease in coupling efficiency and some loss of Ab recognition, particularly in the presence of high molecular weights of mPEG which can sterically hinder access of the Ab to the liposome surface during coupling procedures and/or access of the Ab binding region to its epitope after its attachment to the liposomes [8,18,19]. Because some of the antibody coupling methods in the literature fell considerably short of the ideal, when applied to SL, we have developed a new coupling method, specifically for use with SL, which forms S-immunoliposomes (SIL) by covalently coupling antibodies to the liposomes via a thioether bond at the PEG terminus (Fig. 1), rather than to the phospholipid headgroup region on the liposome surface. This new method comes close to meeting the criteria outlined above for ideal SIL. Attachment of proteins to the distal end of PEG terminus using a PEG-COOH construct has recently been described by two different groups, with retention of long-circulating half-lives and target binding [20,21].

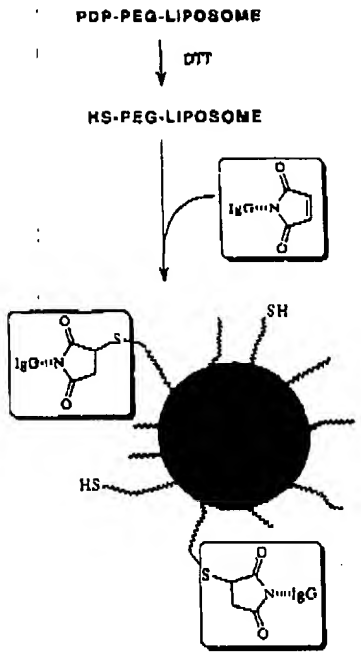

Fig. 1. Schematic diagram of the coupling of a maleimide-activated antibody (MPB-Ab) with sterically stabilized liposomes containing PDP-PEG-DSPE.

2. Materials and methods

2.1. Materials

Distearoylphosphatidylethanolamine (DSPE) was purchased from Sygena, Cambridge, MA, disuccinimidylcarbonate (DSC) from Fluka (Ronkonkoma, NY), and *N*-succinimidyl-3-(2-pyridyldithio)proprionate (SPDP) from Bioaffinity Systems (Rosco, IL). Heterobifunctional α-amino-ω-hydroxy-PEG was prepared by partial conversion of hydroxy end groups of PEG-2000 (Fluka) into primary amines [22], followed by ion-exchange purification according to Furukawa [23]. Amino-PEG-DSPE was prepared as described elsewhere [24].

Hydrogenated soy phosphatidylcholine (HSPC) and methoxy (polyethylene glycol) (2000) distearoylphosphatidylethanolamine (mPEG-DSPE) were obtained from Liposome Technology (Menlo Park, CA). mPEG-DSPE synthesis was described in [14]. Cholesterol (CHOL) and pyridyldithioproprionate dioleoylPE (PDP-DOPE) were purchased from Avanti Polar Lipids (Birmingham, AL). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT); *N*-succinimidyl-4-(*p*-maleimidophenyl)-butyrate (SMPB) and sheep IgG, used as a source of antibody (Ab) were purchased from Sigma (St. Louis, MO). Doxorubicin was obtained from Adria Laboratories (Mississauga, Ontario). Na^{125}I and cholesteryl [1,2(n)-^{3}H]hexadecyl ether, 1.48–2.22 TBq/mmol (^{3}H-CHE) was purchased from New England Nuclear (Mississauga, Ontario). Tyraminylinulin was synthesized and [^{125}I]tyraminylinulin (^{125}I-TI) was prepared as previously described [25]. Iodobeads were purchased from Pierce (Rockford, IL). Monoclonal antibodies (mAb) 174H.64, B43.13 and M170 and the cell lines KLN 205 and Caov-3 were generous gifts of Biomira, Edmonton, AB. The cell line HCT-15 was purchased from American Type Culture Collection (Rockville, MD). All other chemicals were analytical grade.

2.2. Synthesis

General methods

TLC on silica gel G were visualized with iodine vapour, ninhydrin and Dragendorff [26] spray reagents, when ap-

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propriate. NMR spectra was recorded on a 360 MHz Nicolet instrument. When it was important to detect the terminal OH group of PEG, DMSO-d_6 was used as a solvent [27], otherwise $CDCl_3$ was used.

Preparation of PDP-PEG-OH

SPDP (100 mg, 0.32 mmol) and α-amino-ω-hydroxy-PEG (0.55 g, 0.275 mmol) dissolved in acetonitrile (2 ml) reacted at 25° C for 4 h. TLC of the reaction mixture ($CHCl_3/CH_3OH$, 8:2) showed disappearance of H_2N-PEG-OH with appearance of a less polar, UV-absorbing new material ($R_f = 0.80$). The solvent was rotary evaporated and ethyl ether (50 ml) added. After overnight storage at 4° C white solid was collected and dried in vacuo over P_2O_5. Yield: 0.5 g (90%).

H-NMR (DMSO-d_6): $\delta \approx 2.5$ (CH_2S, overlap w/ DMSO), 3.01 (t, $J = 7$ Hz, $CH_2C=O$, 2H), 3.20 (m, CH_2NH, 2H), 3.51 (s, PEG, ≈ 180H), 4.52 (t, HO-PEG, 1H), 7.24 (m, pyr, 1H), 7.76 (m, pyr, 1H), 7.82 (m, pyr, 1H), 7.99 (br m, NH, 1H), 8.45 (m, pyr, 1H) ppm. H-NMR ($CDCl_3$): δ 2.62 (t, $J = 7$ Hz, CH_2SS, 2H), 3.08 (t, $J = 7$ Hz, $CH_2C=O$, 2H), 3.47 (m, CH_2N, 2H), 3.64 (s, PEG, ≈ 180H), 6.78 (br s, NH, 1H), 7.10, (m, pyr, 1H), 7.66 (m, pyr, 2H), 8.49 (m, pyr, 1H) ppm.

Preparation of PDP-PEG-SC

Meticulously dried PDP-PEG-OH (0.4 g, 0.18 mmol) was dissolved in acetonitrile (0.5 ml) and treated with DSC (81 mg, 0.31 mmol) and pyridine (62 ml, 0.79 mmol) overnight at 25° C. The product was precipitated with ethyl ether (40 ml) at 4° C, redissolved in ethyl acetate (4 ml) and precipitated with equal volume of ethyl ether at 4° C. The product was collected by filtration and dried in vacuo over P_2O_5. Yield: 330 mg, (85%).

H-NMR (DMSO-d_6): $\delta \approx 2.5$ (CH_2SS, overlap w/ DMSO), 2.81 (s, SC, 4H), 3.01 (t, $J = 7$ Hz, $CH_2C=O$, 2H), 3.20 (m, CH_2NH, 2H), 3.51 (s, PEG, ≈ 180H), 4.45 (t, CH_2-SC, 2H), 7.24 (m, pyr, 1H), 7.8 (m, pyr, 2H), 8.0 (br m, NH, 1H), 8.45 (m, pyr, 1H) ppm. H-NMR ($CDCl_3$): δ 2.62 (t, $J = 7$ Hz, CH_2SS, 2H), 2.83 (s, SC, 4H), 3.09 (t, $J = 7$ Hz, $CH_2C=O$, 2H), 3.44 (m, CH_2N, 2H), 3.64 (s, PEG, ≈ 180H), 4.46 (m, CH_2-SC, 2H), 6.73 (br s, NH, 1H), 7.10, (m, pyr, 1H), 7.66 (m, pyr, 2H), 8.48 (m, pyr, 1H) ppm.

Preparation of PDP-PEG-DSPE

Method A. DSPE (36 mg, 0.043 mmol) was added to a solution of PDP-PEG-SC (100 mg, 0.042 mmol) in chloroform (1 ml), followed by TEA (33 μl, 0.237 mmol). The reaction mixture became clear during incubation at 40° C for 10 min. The solvent was evaporated and replaced with acetonitrile (5 ml). The cloudy solution was kept at 4° C overnight. To remove traces of insoluble DSPE the solution was centrifuged and the clear solution separated. It was then rotary evaporated under reduced pressure, and the residue was dried in vacuo over P_2O_5. Yield: 130 mg (quantitative).

Method B. To a solution of amino-PEG-DSPE hydrochloride [24] (198 mg, 0.07 mmol) in acetonitrile (2 ml), SPDP (26 mg, 0.085 mmol) was added followed by TEA (30 ml, 0.42 mmol). The solution was stirred overnight. The product (200 mg) in chloroform was loaded onto a silica (11 g) column. It was eluted with a step gradient of CH_3OH in chloroform (5, 10, 15, and 20%, 100 ml each step). Yield: 106 mg of pure product and 77 mg of a product contaminated with a more polar material ($R_f = 0.125$).

The purified products obtained by both methods were identical.

TLC ($CHCl_3/CH_3OH/H_2O$ 90:18:2) $R_f = 0.52$. H-NMR ($CDCl_3$): δ 0.89 (t, $J \approx 6.8$ Hz, CH_3, 6H), 1.26 (s, CH_2, 56H), 1.58 (br m, $CH_2CH_2C=O$, 4H), 2.28 (2 overlapping t, $CH_2C=O$), 2.62 (t, $J = 7$ Hz, CH_2SS, 2H), 3.09 (t, $J = 7$ Hz, $CH_2C=O$, 2H), 3.36 (br m, OCH_2CH_2N, 2H), 3.44 (m, CH_2N, 2H), 3.64 (s, PEG, ≈ 180H), 3.94 (br m, CH_2CH_2OP, 2H), 4.17 (dd, $J = 7.0$, 12 Hz, glycerol CH_2OP, 2H), 4.21 (m, CH_2-O_2CN, 2H), 4.39 (dd, $J = 3.2$, 12 Hz, glycerol $CH_2OC=O$, 2H), 5.20 (m, CH, 1H), 6.73 (br, NH, 1H), 7.10, (m, pyr, 1H), 7.66 (m, pyr, 2H), 8.48 (m, pyr, 1H) ppm. ^{13}C-NMR ($CDCl_3$): δ 14.0 (CH_3), 22.7 (CH_2CH_3), 24.9 ($CH_2CH_2C=O$), 29.7 ($CH_2CH_2CH_2$), 31.9 ($CH_2CH_2CH_3$), 34.1 and 34.3 ($CH_2C=O$), 34.7 ($SCH_2CH_2C=O$), 35.6 ($SCH_2CH_2C=O$), 39.4 ($CH_2NHC=O$), 42.4 (CH_2NHCO_2), 62.8 ($CH_2OC=O$), 63.4 (CH_2OPO_3), 64.2 ($CH_2OC=ON$), 69.9 ($CHOC=O$), 70.6 (PEG), 120.0 (C2 pyr), 137.0 (C3 pyr), 120.8 (C4 pyr), 149.7 (C5 pyr), 156.6 (C = O of urethane), 159.8 (C1 pyr), 170.7 (C = O of amide), 173.0 and 173.3 (C = O of esters) ppm.

2.3. Preparation of immunoliposomes

Liposome preparation

Liposomes were prepared by hydrating dry lipid films in an appropriate buffer, at a lipid concentration of 10 mM. The resulting multivesicular preparations were then passed through 0.08–0.1 μm polycarbonate membranes (Nuclepore, Pleasanton, CA or Poretics, Livermore, CA) using a Lipex extruder (Lipex Biomembranes, Vancouver, BC), to give primarily unilamellar vesicles of approx. 100 nm in diameter [28,29]. The resulting liposomes were sized by dynamic light scattering using a Brookhaven BI90 particle sizer (Brookhaven Instruments, Holtsville, NY).

Iodination of antibody

Ab (2 mg) was dissolved in 200 μl of 25 mM Hepes, 140 mM NaCl, pH 7.4 buffer. The Ab solution was mixed with 185 MBq of $Na^{125}I$ in a 2 ml reaction vial with 5 Iodobeads for 1 h at room temperature (22° C). ^{125}I-Ab was purified by passage over a Sephadex G25 gel filtration column, eluting with the above buffer.

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Preparation of maleimidophenylbutyrate-Ab (MPB-AB)

Ab was dissolved in 25 mM Hepes, 140 mM NaCl, pH 7.4 at concentration of 10 mg/ml and trace amounts of ^{125}I-Ab were added. SMPB (25 mM in dimethylformamide) was slowly added to the Ab solution at a molar ratio of 20:1 (SMPB/Ab) for 30 min at room temperature. Unbound SMPB was removed and the pH lowered by passing the solution over a Sephadex G50 column in 25 mM Hepes, 25 mM Mes, 140 mM NaCl, pH 6.7 buffer.

Antibody conjugation

Liposomes were composed of HSPC/CHOL/PDP-PEG-DSPE at a molar ratio of 2:1:0.02 with or without 4 mol% mPEG-DSPE (total PEG lipid, 5 mol% of PL) or of HSPC/CHOL/PDP-DOPE, at a molar ratio of 2:1:0.02 with or without 5 mol% mPEG-DSPE. In experiments to determine the coupling efficiency of the PDP-PEG-PE method, the ratio of PDP-PEG-DSPE and mPEG-DSPE were varied, keeping the total amount of PEG-lipids in the liposomes constant at 5 mole% of phospholipids (PL). Trace amounts of ^{3}H-CHE was added to each liposome preparation and the PL concentration was calculated from the specific activity using a Beckman LS6800 Scintillation counter.

Liposomes were hydrated with 100 mM sodium acetate, 70 mM NaCl, pH 5.5 buffer. The pyridyldithio groups were reduced by the addition of dithiothreitol (DTT) to a final concentration of 20 mM for 30 min at room temperature. DTT was separated and the pH raised by passing the liposomes over a Sephadex G50 column eluted with 25 mM Hepes, 25 mM Mes, 140 mM NaCl, pH 6.7 buffer.

Thiolated liposomes (2 μmol PL) were incubated overnight at room temperature with MPB-Ab (0.25–8 nmol) at Ab/PL molar ratios ranging from 1:250 to 1:8000, at a final PL concentration of 2–4 mM. Unbound Ab was removed by passing the liposomes over a Sepharose CL-4B column with pH 7.4 buffer (25 mM Hepes, 140 mM NaCl). The Ab to PL ratio was determined by counting for ^{125}I-IgG and ^{3}H-CHE. The amount of ^{125}I in the ^{3}H channel was subtracted from the total ^{3}H counted and this overlap was kept below 10% of ^{3}H.

2.4. Properties of immunoliposomes

Liposomes were composed of HSPC/CHOL/mPEG-DSPE/PDP-PEG-DSPE (2:1:0.08:0.02 molar ratio) or HSPC/CHOL/PDP-DOPE (2:1:0.02 molar ratio). Liposomes were hydrated with the aqueous space label ^{125}I-TI [30] and prepared as above, with diameters of 98–130 nm. Free ^{125}I-TI was separated from the liposomes by chromatography over a Ultragel AcA 34 column (IBF Biotechnics, France) with 25 mM Hepes, 140 mM NaCl, pH 7.4 buffer. MPB-Ab was then conjugated to PDP-containing liposomes at various ratios to give Ab surface densities of 0–140 μg Ab/μmol PL.

In these experiments, the amount of Ab bound to the liposomes was determined by an adaptation of a fluorescamine assay, where an increase in fluorescence is observed when fluorescamine binds to a free amino group on a protein [31,32]. Briefly, 10 mg fluorescamine was dissolved in 50 ml of dry acetonitrile. One ml samples of SIL (0.2–1 mM PL) were mixed with 1 ml of fresh fluorescamine reagent for 15 min at room temp then 1 ml of ethanol was added. The relative fluorescence was determined on a Perkin-Elmer MPF-4 spectrofluorimeter (EM 475 nm, EX 395 nm). Protein concentrations were determined from a standard curve using known concentrations of MPB-Ab (5–160 μg/ml).

Outbred female CD$_1$(ICR)BR mice were purchased from Charles River Canada (St. Constant, Quebec) and maintained in standard housing. Mice (three per group) were given a single bolus i.v. injection via the tail vein of 0.2 ml of SIL (0.5 μmol PL/mouse) containing $(1–3)\cdot 10^{5}$ cpm of ^{125}I-TI. At different times post-injection, animals were anaesthetized with halothane and sacrificed by cervical dislocation. Major organs (liver, spleen, lung, heart and kidney), blood (100 μl), thyroid and carcass (remainder of the animal) were collected and counted for ^{125}I label in a Beckman 8000 gamma counter. Blood correction factors [33], were applied to all samples. The data is expressed as the percentage of counts in each organ relative to the total counts remaining in vivo at each time point [30].

The blood elimination half life of SIL was calculated using the polyexponential curve stripping and least squares parameter estimation program RSTRIP (Micromath, Salt Lake City, USA).

Doxorubicin loading and leakage studies

For DXR loading experiments, liposomes were hydrated in 155 mM ammonium sulfate at pH 5.5 [34]. The liposomes were then passed over a Sephadex G50 column equilibrated in 123 mM sodium citrate, pH 5.5. DXR (0.2 mg/mg PL) was then added to the liposomes and the mixture was incubated at 65° C for 1 h. DXR-containing liposomes were separated from any remaining free DXR by passage over a Sephadex G50 column and eluted with 123 mM sodium citrate pH 5.5 buffer.

For leakage experiments, DXR-loaded liposomes, composed of HSPC/CHOL/mPEG-DSPE/PDP-PEG-DSPE (2:1:0.08:0.02 molar ratio), were labelled with trace amounts of ^{3}H-CHE. Liposomes were coupled with ^{125}I-Sheep IgG (SIL[sheep IgG]) to give 88 μg Ab/μmol PL, then mixed with 25% human plasma in 25 mM Hepes, 140 mM NaCl, pH 7.4 at 0.6–1.2 mM PL and dialysed (Spectra/Por 2, 12 000–14 000 M_r cut-off; Spectrum, Los Angeles, Ca) against 50 volumes of 25% human plasma. At various times, samples were removed and the external plasma volume exchanged. The amount of DXR remaining in the liposomes was determined by extracting the DXR in methanol and determining the absorbance at A_{492} nm.

Uptake and cytotoxicity studies

Murine squamous lung carcinoma (KLN 205), human ovarian adenocarcinoma (Caov-3), and human colon

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adenocarcinoma (HCT-15) cell lines were grown as monolayers in RPMI 1640 supplemented with 10% fetal bovine serum (Gibco BRL, Burlington, Ontario, Canada) at 37° C in a humidified atmosphere of 5% CO_2 in air. Cells were plated in triplicate onto 6-well plates (Falcon, Becton Dickinson, Lincoln Park, NJ) at $(0.15–1)\cdot 10^6$ cells/well on day 1. SIL were prepared by conjugating (see above), to the surface of SL (HSPC/CHOL/mPEG-DSPE/PDP-PEG-DSPE, 2:1:0.08:0.02 molar ratio), mAb specific to each cell line, i.e., mAb 174H.64, B43.13 or M170 for experiments involving KLN 205, Caov-3 or HCT-15, respectively. On the fourth day (or at the point where the cells just reach confluence), SIL[174H.64], SIL[B43.13] or SIL[M170], labelled with ^{3}H-CHE (0.1–0.4 μmol/ml), in PBS were added to each well of their targeted cell lines, respectively. In some instances, binding of SIL to cells was also measured in the presence of 10% FBS. After 1 h incubation at 37° C, cells were washed three times with phosphate-buffered saline, pH 7.4, trypsinized with 0.5 ml of 0.05% trypsin, placed in ACS scintillation fluid (Amersham, Oakville, Ontario), and counted in a Beckman LS-6800 counter.

A colorimetric assay using a tetrazolium salt was used for the measurement of surviving and/or proliferating cells according to Mosmann [35]. Cells (KLN 205) were plated in 96-well plates (Corning, Corning, NY) on day 1. On the third day, cells were incubated with either free DXR, DXR entrapped in non-targeted liposomes (DXR-SL) or DXR entrapped in SIL[174H.64] (DXR-SIL[174H.64]) and the cells were incubated for 1 h or 24 h at 37° C in a humidified 5% CO_2 atmosphere. At the end of the incubation, free or liposomal DXR was removed by gentle washing with phosphate-buffered saline, pH 7.4, and the cells were further incubated for 47 h or 24 h respectively, for a total of 48 h. At the end of the incubation, a solution of MTT (0.5 mg MTT/ml media) was added to each well, and the mixture was incubated at 37° C for 4 h. Acid-isopropanol (100 μl of 0.04 M HCl in isopropanol) was added to each well and mixed thoroughly until all crystals were dissolved. The plates were read immediately on a Titertek Multiskan PLUS MK II plate reader (Flow Laboratories, Mississauga, Ontario, Canada), using a test wavelength of 570 nm, and a reference wavelength of 650 nm.

3. Results and discussion

3.1. Synthesis of PDP-PEG-DSPE

For preparation of SILs we chose the conjugation strategy shown in Fig. 1 for the following reasons. Reaction between thiol and maleimide groups is one of the most useful and efficient reactions in bioconjugate chemistry [6,36]. It takes place at close to neutral pH, at ambient temperature, within short periods of time, and often results in satisfactory yields of conjugates, even when relatively

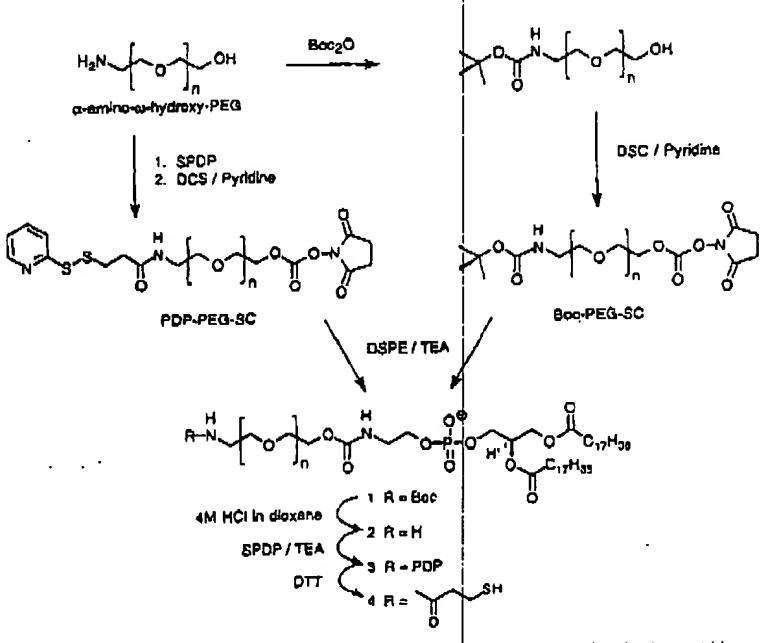

Fig. 2. Synthetic pathways for PDP-PEG-DSPE. Both PDP-PEG-DSPE synthetic pathways started with the heterobifunctional polymer, α-amino-ω-hydroxy poly(ethylene glycol) [24]. On the right, α-amino-ω-hydroxy-PEG is coupled with SPDP then with DSPE. On the left, α-amino-ω-hydroxy-PEG is first protected with Boc then bound to DSPE and subsequently Boc was removed and replaced with SPDP. Once incorporated into liposomes containing derivative 4, a free thiol was generated by reduction of PDP with DTT (see Materials and methods).

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low concentrations of the reactants are present. Thus it seems to be particularly suitable for interlinking of two macromolecular entities.

Previous experience with maleimide-PE incorporated into liposomes showed that it interfered with the drug loading procedures [8]. Maleimide is also known to undergo gradual degradation under conditions used during liposome preparation, sizing, and drug loading steps [6,37]. On the other hand, a maleimide group can be introduced onto IgG just prior to the actual conjugation step giving it no opportunity to undergo any side reactions.

Thiol groups, while very reactive towards various electrophiles, can be preserved under non-oxidizing conditions. To absolutely assure the integrity of the thiol groups, we decided to prepare a protected form of thiol group (PDP) positioned at the terminus of PEG-DSPE. Thus, our strategy involved preparation of liposomes containing the new conjugate, PDP-PEG-DSPE, first. They were conveniently formulated and drug-loaded, and after reductive deprotection of the reactive thiol groups with DTT, conjugated with maleimide-containing IgG (see Fig. 1).

The two synthetic pathways leading to PDP-PEG-DSPE are schematically depicted in Fig. 2. The synthesis started with heterobifunctional polymer, α-amino-ω-hydroxy poly(ethylene glycol), which was prepared by partial conversion of the hydroxyl end groups into primary amines [22] followed by ion-exchange purification of H_2N-PEG-OH [23]. The amino group was selectively acylated with di-*tert*-butyldicarbonate resulting in the introduction of Boc protecting group. Proton-NMR obtained in DMSO-d_6 confirmed the presence of the protecting group as well as the unaltered hydroxyl. The remaining three steps of the synthesis were performed similarly to the previously published method for preparation of hydrazido-PEG-DSPE [39]. Briefly, a succinimidylcarbonate (SC) group, introduced at the hydroxy-end of the polymer, was used to form urethane attachment of the polymer to the amino group of DSPE yielding Boc-protected amino-PEG-DSPE (1) at the end of the PEG chain. The primary amine functionality was regenerated by acidolytic removal of the Boc-group forming 2. Finally, PDP-PEG-DSPE (3) was obtained by acylation of the terminal amino group with SPDP.

Alternatively α-amino-ω-hydroxy-PEG could be directly reacted with SPDP substituting only the amino group. Then the remaining hydroxy end group was cleanly converted into succinimidyl carbonate and the resulting macromolecular analog of SPDP (PDP-PEG-SC) was coupled to DSPE. It appears that the second approach is preferable for a direct preparation of PDP-PEG-DSPE (3). It is two steps shorter and it resulted in higher yields of the product. The first approach is more general and allows conversion of amino-PEG-DSPE [24] into various functionalized PEG-DSPE-derivatized conjugates via use of different heterobifunctional reagents [38].

It is important to note that the utility of the new macromolecular crosslinker, PDP-PEG-SC clearly extends beyond the current application. It is in a sense a PEG analog of SPDP and as such offers several advantages. Unlike SPDP, PDP-PEG-SC is water soluble. Since, PEG is a material of choice for rendering surfaces biocompatible, PDP-PEG-SC is perfectly suitable for linking ligands to biomaterials intended for blood plasma contact. It is ideally suited for preparation of two or more component macromolecular conjugates, e.g., protein-protein crosslinking. In this case, the PEG spacer, being very flexible and well solvated in water, would allow maximum freedom of mobility for each of the conjugated components.

Table 1
Coupling efficiency and Ab density in liposomes containing PDP-PEG-DSPE

mol% mPEG-DSPE	mol% PDP-PEG-DSPE	Initial Ab/PL molar ratio	Ab density (μg Ab/μmol PL)	Coupling efficiency (%)
3	2	1: 250	316	51
		1: 500	265	85
		1:1000	136	87
4	1	1:1000	114	73
		1:2000	63	81
4.5	0.5	1: 500	82	26
		1:1000	76	48
		1:2000	66	85
4.75	0.25	1: 500	41	13
		1:1000	36	23
		1:2000	35	45
5	0	1: 500	11	3.4
		1:1000	10	6.1
		1:2000	7	9.5
0	1	1:1000	93	60
		1:2000	69	88

Liposomes were composed of HSPC/CHOL, 2:1 molar ratio and contained 5 mol% total PEG, consisting of a combination of mPEG-DSPE and PDP-PEG-DSPE as indicated. Liposomes averaged 100 nm in diameter. The source of Ab was sheep IgG and the Ab/PL molar ratio in the coupling mixture (2 mM PL) was varied from 1:250 to 1:2000. The coupling procedure, schematically shown in Fig. 1, is described in detail in Materials and methods. The coupling efficiency is expressed as the % of initial Ab attached to the liposomes.

3.2. Coupling of antibodies to liposomes containing PDP-PEG-DSPE

The PDP-PEG-DSPE could be incorporated easily into liposomes during their formation and Ab could be efficiently coupled to the surface of liposomes containing PDP-PEG-DSPE following the reduction of the PDP group with DTT. A comparison of the effect of the PDP-PEG-DSPE content in the liposomes, and the effect of Ab/PL ratio on coupling efficiency and the Ab density conjugated at the liposome surface is given in Table 1. Higher mol% of PDP-PEG-DSPE incorporated into the liposomes resulted in higher Ab densities at the liposome surface, with the highest densities being over 300 μg Ab/μmol PL (Table 1) corresponding to approx. 150 Ab/100 nm diameter liposome, which is almost complete coverage of the

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surface of the liposome. One can estimate that, at complete coverage, 200 Ab can be bound to a liposome 100 nm in diameter. These calculations were based on the following assumptions: the effective diameter of a IgG_1 molecule is 142 Å [40] (150 000 Da), the area per polar head for HSPC is 72 $Å^2$ and for CHOL is 19 $Å^2$ [41] with an combined area per phospholipid of 81.5 $Å^2$ for a HSPC/CHOL (2:1 mol/mol) liposome.

As the amount of PDP-PEG-DSPE in the liposomes was decreased, the Ab density at the liposome surface also decreased, but even at the lowest concentration of PDP-PEG-DSPE (0.25 mol%), substantial amounts of Ab still could be conjugated to the liposome surface (35–41 μg Ab/μmol PL) (Table 1). In the absence of PDP-PEG-DSPE, very low amounts of Ab were associated with the liposomes (approx. 10 μg Ab/μmol PL), possibly through passive absorption (Table 1).

When the initial Ab/PL molar ratios were kept constant (e.g., 1:1000), the coupling efficiency decreased as the concentration of PDP-PEG-DSPE in the liposomes decreased, from a high of 87% of the Ab attached to liposomes in the presence of 2 mol% PDP-PEG-DSPE to a low of 23% in the presence of 0.25 mol% PDP-PEG-DSPE (Table 1). Although Ab density decreased as the initial Ab/PL molar ratio decreased, the coupling efficiency increased as this ratio decreased. A plot of coupling efficiency versus PDP-PEG-DSPE/Ab ratio demonstrates that the % of initial Ab bound to the liposomes reaches a maximum of greater than 80% above PDP-PEG-DSPE/Ab ratios of 10:1 (Fig. 3). In other words, for maximum coupling efficiency to occur, the PDP-PEG-DSPE must be present in approximately a 10-fold excess.

In the absence of mPEG-DSPE in the liposomes, equivalent amounts of Ab became associated with the liposomes as in the presence of mPEG-DSPE, with similar coupling efficiencies (Table 1). This demonstrates that the Ab can gain easy access to its coupling site at the PEG terminus.

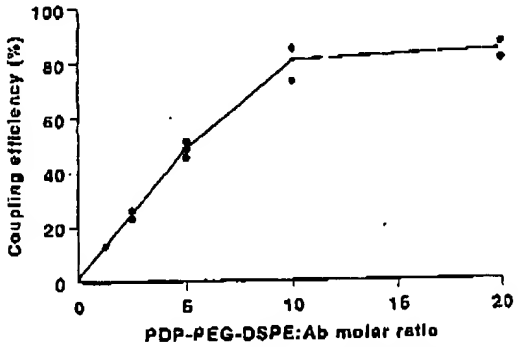

Fig. 3. Coupling efficiencies as a function of PDP-PEG-DSPE/Ab molar ratio. Liposomes (100 nm in diameter), composed of HSPC/CHOL/mPEG-DSPE/PDP-PEG-DSPE (2:1:0.08:0.02 molar ratio), were coupled to Ab (sheep IgG) at various molar ratios of PDP-PEG-DSPE/Ab (2 mM phospholipid). The coupling efficiency is expressed as the % of initial Ab bound to the liposomes.

Table 2
Coupling efficiency and Ab densities for liposomes containing PDP-DOPE

Initial Ab/PL molar ratio	Ab density (μg Ab/μmol PL)	Coupling efficiency (%)
1: 500	28	9
1:1000	15	10
1:2000	7.4	9

Liposomes were composed of HSPC/CHOL, 2:1 molar ratio and contained 5 mol% mPEG-DSPE and 1 mol% PDP-DOPE. Liposomes averaged 100 nm in diameter. The source of Ab was sheep IgG and the Ab/PL molar ratio in the coupling mixture (2 mM PL) was varied from 1:500 to 1:2000. The coupling procedure was as outlined in Materials and methods. The coupling efficiency is expressed as the % of initial Ab attached to the liposomes.

This is in contrast to methods where Ab is coupled at the liposome surface when mPEG can sterically interfere with access of Ab to its coupling site [8]. Further data in support of this conclusion is presented in Table 2. For liposomes containing PDP-DOPE and mPEG-DSPE (i.e., the PDP group is at the liposome surface), the coupling efficiency and Ab densities are significantly lower than for liposomes containing PDP-PEG-DSPE. We suggest that this is due to steric hinderance by mPEG-DSPE to access of maleimide-Ab to the reduced PDP groups (thiol-PE) at the liposome surface.

3.3. Remote loading of doxorubicin and drug leakage rates

In the PDP-PEG-PE coupling method, the MPB group is attached to the Ab rather than to the liposome surface, as in coupling methods using MPB-PE [6,7]. Hence the MPB group is present in much lower concentrations, and is located much further from the immunoliposome surface after conjugation, than in the MPB-PE method. DXR could be remote loaded into SL (HSPC/CHOL/mPEG-DSPE/PDP-PEG-DSPE, 2:1:0.08:0.02) prior to Ab conjugation with greater than 95% efficiency after 1 h at 65° C. The half-life for release of doxorubicin from these liposomes, following conjugation of Ab was in excess of 150 h. This is in contrast to the difficulties with the remote loading of doxorubicin into liposomes containing the MPB group at the liposome surface (MPB-PE method), where the presence of the hydrophobic MPB group at the liposome surface appears to significantly decrease the rate of loading of doxorubicin and increase the rate of drug leakage [8].

3.4. Pharmacokinetics of S-immunoliposomes formed by the PDP-PEG-DSPE method

Immunoliposome clearance was determined with liposomes containing the aqueous space label, ^{125}I-tyraminyl-inulin (^{125}I-TI). Free ^{125}I-TI was removed from the body with a $t_{1/2}$ of a few minutes, while the liposome-entrapped label had a long latency time in the body with a $t_{1/2}$ of 78

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h, indicating that the label had a very slow leakage rate from the liposomes in vivo. Leakage of the label was totally independent of the presence of Ab on the liposomes. The blood levels, as a function of time, for SIL containing increasing Ab densities (sheep IgG), formed by the PDP-PEG-PE method, are shown in Fig. 4A and B. Polyclonal sheep IgG was used for these experiments in order to not to deplete our limited supplies of monoclonal antibodies, however in our experience, there is no difference in the pharmacokinetics between polyclonal and monoclonal antibodies.

The addition of PDP-PEG-DSPE into S-liposomes (no Ab) did not significantly alter their circulation times ($t_{1/2}$ of 16 h, with greater than 80% of the label remaining liposome entrapped and therefore remaining in vivo). The SIL were removed from circulation in a log-linear manner at Ab densities of 74 μg/μmol PL and lower (Fig. 4A) and the level of SIL in blood at 24 h decreased with an increase in the Ab density (Fig. 4B). High Ab densities (140 μg/μmol PL) resulted in rapid removal of the SIL from circulation (Fig. 4A and B), but no change in the rate of label leakage from the liposomes. Ab densities in the range of approx. 20–80 μAb/μmol PL resulted in $t_{1/2}$ in the circulation of from 7 to 9 h compared to 16 h in the absence of Ab (with greater than 90% of the entrapped liposome label remaining in vivo), which is likely to be sufficiently long to allow in vivo targeting. By contrast, liposomes lacking mPEG (73 μg Ab/μmol PL) were rapidly removed from circulation (Fig. 4A) even at low Ab densities, with half-lives of less than 0.5 h.

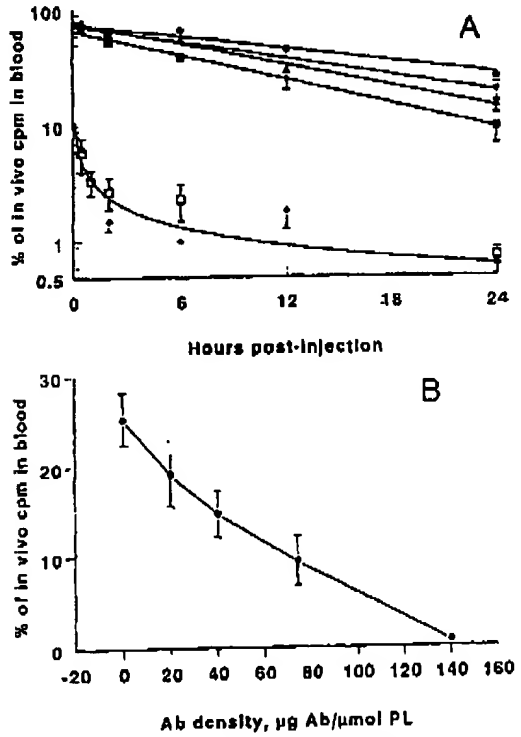

Fig. 4. Blood clearance kinetics of S-immunoliposomes in mice. Antibody (sheep IgG) was coupled at various densities to liposomes (HSPC/CHOL/PDP-PEG-DSPE, 2:1:0.02 molar ratio ±4 mol% mPEG-DSPE, 100 nm diameter) containing the aqueous space label ^{123}I-TI. The resulting S-immunoliposomes were injected i.v., via the tail vein, into CD_1 (ICR) mice. Results are expressed as label remaining in blood as a percentage of the label remaining in the body at various times post injection (means ± S.D., $n = 3$). (A) Blood clearance of S-immunoliposomes as a function of time and Ab density. Liposomes contained various Ab densities in the presence of mPEG-DSPE (●) no Ab; (▼) 20 μg Ab/μmol PL; (▲) 40 μg Ab/μmol PL; (■) 74 μg Ab/μmol PL; (♦) 140 μg Ab/μmol PL. Control liposomes (◆) contained no mPEG-DSPE, and had an antibody density of 73 μg Ab/μmol PL. (B) Blood clearance of SIL at 24 h post-injection as a function of Ab density

3.5. Binding to, and cytotoxicity to, cancer cells in culture by S-immunoliposomes

The binding by three different cancer cell lines, of SIL conjugated by the PDP-PEG-PE method to three different mAb specific for each cell line, is shown in Fig. 5. In each case the attachment of a specific mAb to SIL (SIL[174H.64], SIL[B43.13] or SIL[M170]) resulted in 2-to 3-fold increased binding of the SIL to their respective cell lines (KLN-205, Fig. 5A; Cnov.3, Fig. 5B; or HCT-15, Fig. 5C). Increasing the Ab density at the liposome surface resulted in a modest increase in the amount of SIL bound to the KLN-205 cells (Fig. 5A). We have previously shown that an isotype matched non-specific Ab (B27.29, Biomira) conjugated to immunoliposomes by the PDP-PEG-PE method showed no increase in binding to KLN-205 cells compared with antibody-free liposomes [42,43]. Excess free mAb 174H.64 would compete for binding of SIL[174H.64] to KLN-205 cells, but free mAb B27.29 was not effective in displacing binding of SIL[174H.64] [42]. Incubation of HCT-15 cells in the presence of 10% FBS reduced the binding of SL (no mAb) and SIL[M170] to the cells by approx. 2-fold, but the increase in binding of SIL compared with SL was, if anything, greater in the presence of serum than in its absence (Fig. 5D versus 5C).

The ability of SIL[174H.64] to increase the cytotoxicity of entrapped DXR to KLN-205 cells was also examined (Table 3). The IC_{50} for DXR-SIL[174H.64] at 1 h incubation was lower than that for either free DXR or DXR-SL. For this short incubation time, the free DXR and DXR-SL, both of which would be expected to have only low levels of non-specific association with the cells, were likely washed away before the drug could be taken up into cells in significant amounts. In addition, the very high IC_{50} for DXR-SL is likely also due to the sequestration of the DXR inside liposomes, with relatively little DXR released from the liposomes during the 1 h incubation period. As anticipated, free DXR and DXR-SL had significantly lower IC_{50} after a 24 h incubation, while the SIL[174H.64] appeared to have reached close to its maximum cytotoxicity after 1 h incubation and only experienced a slight decrease in IC_{50}

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Table 3
Cytotoxicity of SIL[174H.64] against murine squamous lung carcinoma cells in culture

Sample	IC_{50}, μg DXR/ml of media for KLN 205 cells	
	1 h incubation	24 h incubation
Free DXR	93	15
DXR-SL	> 200	68
DXR-SIL[174H.64]	42	31

Murine squamous lung carcinoma (KLN 205) cells were plated in 96-well plates on day 1, 24 h later, free DXR, DXR-SL or DXR-SIL[174H.64] was incubated with the cells for 1 h or 24 h prior to gently washing the cells three times with phosphate-buffered saline, pH 7.4. The cells were then incubated for a further 47 or 24 h, respectively, (total incubation time, 48 h). The IC_{50} (μg DXR/ml) was determined from cell viability using a tetrazolium dye assay [35].

after 24 h. At 24 h, SIL[174H.64] were slightly less cytotoxic than free DXR, although more cytotoxic than DXR-SL. It would appear that the 1 h incubation period was sufficient for binding and/or internalization of the SIL[174H.64] to liposomes, as the longer incubation time did not result in very large increases in cytotoxicity for these liposomes. The 1 h incubation would be more similar to the in vivo situation, where free DXR would be rapidly distributed throughout the body in a large volume of distribution [44,45], with tumor cells exposed to relatively low concentrations of the drug. The SIL, on the other hand, when bound to tumor cells in vivo, might be expected to deliver higher amounts of drug to the tumor in a sustained manner, either through release of locally high concentrations of drug from SIL bound at the tumor surface and uptake of DXR through the normal uptake mechanisms, or through Ab-mediated internalization of the drug-liposome package. At present we are undertaking experiments to distinguish between the relative contribution of these two mechanisms of SIL-mediated cytotoxicity, however, independent of the mechanism of cytotoxicity, we have recently demonstrated a dramatic increase in mean survival time of mice inoculated with the KLN-205 cell line and treated with DXR-SIL[174H.64] relative to DXR-SL or free DXR [46].

In conclusion, we developed a simple 3 step synthesis of new end-group functionalized PEG-lipid, PDP-PEG-DSPE, starting from α-amino-ω-hydroxy-PEG. Incorpora-

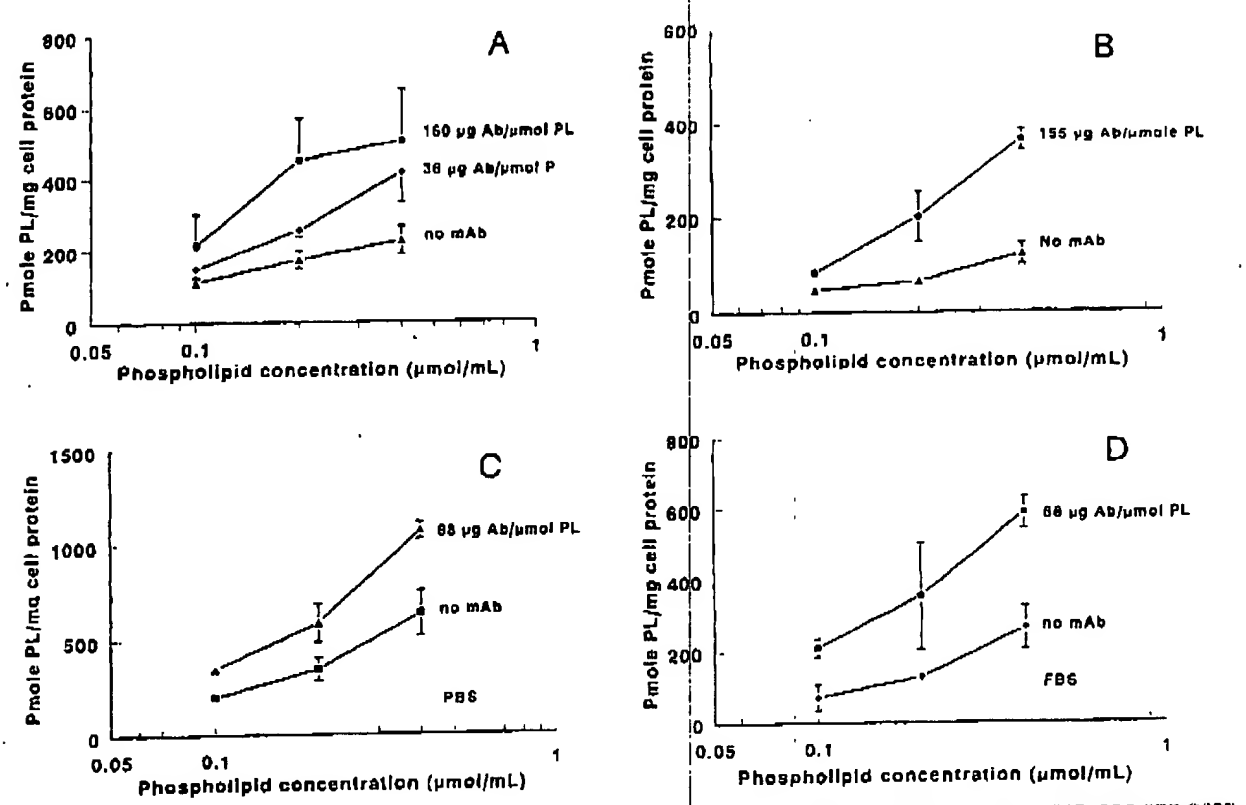

Fig. 5. Binding of S-immunoliposomes by various tumor cell lines. S-immunoliposomes in PBS (HSPC/CHOL/mPEG-DSPE/PDP-PEG-DSPE. 2:1:0.08:0.02 molar ratio, 100 nm in diameter) were labelled with ^{3}H-CHE and incubated for 1 h at 37°C with cells at a liposome concentration of 0.1–0.4 μmol PL/ml. (A) Murine squamous lung carcinoma (KLN205) and mAb 174H.64 coupled to SIL; (B) Human ovarian adenocarcinoma (Ca$_{ov}$-3) and mAb B43.13 coupled to SIL; (C) human colon adenocarcinoma (HCT-15) and mAb M170 coupled to SIL. (D) HCT-15 cells and mAb M170 coupled to SIL incubated in the presence of 10% FBS. means ± S.D., $n = 3$.

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tion of this PEG-lipid conjugate into liposomes allows for convenient generation of thiol groups at the distal ends of the grafted PEG chains. Incubation of the vesicles containing thiol-PEG-DSPE (4) with maleimide-antibodies resulted in efficient conjugation even when content of the functionalized PEG-DSPE component in the liposomes was below 1 mol%. This method realizes many of the criteria which would be desirable for 'ideal' S-immunoliposomes including simplicity, high coupling efficiency, the ability to achieve a large range of Ab densities at the liposome surface, significantly increased survival times in circulation compared to classical immunoliposomes, efficient drug loading, slow drug release rates, and retention of target binding.

Acknowledgements

This work was supported by MRC Canada (UI-12411) and Liposome Technology Inc., Menlo Park, CA. We thank Biomira Inc., Edmonton for their generous donation of monoclonal antibodies and cell lines for these experiments.

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